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Using Embryonic Stem Cells as a Novel Model to Compare the Toxicological Effects of Harm Reduction and Conventional Cigarette Smoke on Early Embryo Development

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Using Embryonic Stem Cells as a Novel Model to Compare the Toxicological Effects of Harm Reduction and Conventional Cigarette Smoke on Early Embryo Development

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

by

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June 2010

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ABSTRACT OF DISSERTATION

Using Embryonic Stem Cells as a Novel Model to Compare the Toxicological Effects of Harm Reduction and Conventional Cigarette Smoke on Early Embryo Development

by

Sabrina Chia-Chin Lin

Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology
University of California, Riverside, June 2010
Dr. Prue Talbot, Chairperson

Embryonic stem cells are derived from the inner cell mass (ICM) of blastocysts. Before implantation, cells in the ICM undergo multiple divisions, and after implantation, the entire ICM develops into the embryo. Here, we are interested in the effects of environmental toxicants, such as cigarette smoke, on blastocysts during pre-implantation development. Our lab used mouse and human embryonic stem cells (mESC, hESC) as a pre-implantation model to examine the effects of mainstream (MS) or sidestream (SS) smoke from conventional (Marlboro Red) and harm reduction (Advance Lights, Quest, and Marlboro Lights) cigarettes on early development. Harm reduction tobacco products, contain genetically modified tobacco to produce lower nicotine concentration or are equipped with advanced filters designed to remove carcinogens. These products are often advertised as safer with fewer toxins. In our mESC study, stem cells were cultured in suspension
with cigarette smoke solutions and allowed to attach over 24 hours. All brands tested inhibited mESC attachment, survival, and proliferation, and surprisingly, harm reduction cigarette smoke was more potent than conventional smoke (Lin et al., 2009). To further investigate the effects of cigarette products on human embryos, hESC colonies were treated with conventional and harm reduction cigarette smoke solutions for 48 hours. To overcome technical challenges with the hESC system, BioStation technology combined with video bioinformatics tools was used to develop assays based time-lapse video data of dynamic cellular processes (Lin, et al., 2010). This technology was used to quantify hESC colony attachment and growth in various smoke treatments. Data demonstrated that non-cytotoxic doses of conventional and harm reduction cigarette SS smoke impaired hESC colony attachment and growth significantly, but MS smoke did not. Moreover, hESC were more sensitive in most assays than mESC. Most significantly, for both species in all assays, SS smoke from harm reduction cigarettes was more potent than SS smoke from a conventional brand. This study clearly demonstrates the need to monitor harm reduction products carefully and to evaluate both MS and SS smoke emitted from them.
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CHAPTER 1

INTRODUCTION
MOUSE AND HUMAN EMBRYONIC STEM CELLS: CAN THEY IMPROVE HUMAN HEALTH BY PREVENTING DISEASE?
Abstract

Given the vast number of chemicals that are released into the environment each year, it is imperative that we develop new predictive models to identify toxicants before unavoidable exposure harms the health of humans and other organisms. *In vitro* models are especially attractive in predictive toxicology as they can greatly reduce assay costs and animal usage while identifying those chemicals that may require further *in vivo* evaluation. With the derivation of both mouse and human embryonic stem cells, new opportunities have developed that could revolutionize the field of predictive toxicology. Stem cells themselves can be used to model pre-implantation development, or they can be used during or after differentiation and thereby model post-implantation development. Because embryos and fetuses are usually the more sensitive to environmental toxicants than adults, stem cells provide an unique tool for studying the prenatal phase in our life cycle. The embryonic stem cell test (EST), which has been validated for use with mouse ESC (mESC), is an accurate predictor of embryotoxic compounds, particularly those that are highly embryotoxic. Human embryonic stem cells (hESCs), although not yet incorporated into a validated test, are a particularly attractive platform for toxicological testing as they can give us direct information on humans and avoid the concern about species variation in response. This review discusses toxicological studies and strategies that have been used with embryonic stem cells during the past five years and possible directions that could lead to improvements in the development of predictive assays in the future.
**Introduction**

The United States releases over 1,500 new chemicals into the environment each year, and this number is much higher worldwide\textsuperscript{1}. Most of these chemicals have not undergone adequate toxicological testing prior to their release, and their potential to adversely affect human health is largely unknown. In addition, drug companies screen numerous chemicals each year for toxic or undesired side effects prior to developing them into potential products. Our ability to monitor and identify toxic chemicals and drugs before they cause harm to humans and other species has been important for many years, and numerous strategies have been developed to deal with this problem. However, methods to evaluate the toxicity of both environmental chemicals and potential drugs need to be modernized and improved to deal with the growing need to protect human health yet make sophisticated progress toward development of new industrial products and medicines. In developing new assays, it is important to consider that the unborn are generally the most sensitive to chemical exposure, and leading toxicologists recently advocated that future evaluation of chemicals be done on prenatal stages of development so that the most vulnerable in our population will be protected from exposure\textsuperscript{2}. Most chemical testing is currently done using laboratory animal models, usually mammals such as rats or mice or non-mammalian species such as the zebrafish\textsuperscript{3}. The cost of performing animal studies is very high, requires a large number of animals for reliable data, often requires a significant amount of time to complete, and is based on non-human species that may not respond the same way to a test
chemical as humans. There is clearly a need to develop new methods for evaluating toxicity without complete reliance on animal testing.

The value of using in vitro models for measuring toxicity of environmental chemicals and drugs has been appreciated for many years and discussed in recent reviews. In contrast to laborious in vivo models that require many animals and may not be accurate predictors for humans, in vitro models potentially enable high throughput screening of chemicals and drugs and can be done using human cells, which should be better predictors of human health effects than models based on non-human species. Chemicals found to be toxic in vitro in initial screens could be further evaluated in more elaborate in vitro assays, such as metabolizing assays, or in vivo in an animal model. Moreover, in vitro studies are generally less expensive and may be conducted more rapidly than experiments in animal models. Numerous in vitro models using both mammalian and non-mammalian sources have been developed over the past 30 years. In general, these have been good predictors of developmental toxicity, although they vary considerably in the time and difficulty required to perform them. With the introduction of mouse embryonic stem cells in 1981, it became apparent that these cells could be an excellent model for early embryonic development, and they have subsequently been used in toxicological testing. Human embryonic stem cells, which were first derived in 1998, extended the opportunity to develop in vitro assays based on human cells that model embryonic development. The purpose of this review is to summarize and discuss
work that has been achieved in developing and using embryonic stem cells in toxicological testing during the past 5 years.

**Embryonic Stem Cells (ESC) – what are they?** All stem cells have the ability to self renew indefinitely and to produce daughter cells that can differentiate into another type of cell(s). Stem cells come from various sources, and they are usually named according to their source. For example, stem cells that are isolated from the inner cell mass of blastocysts are called embryonic stem cells (ESC), while cells isolated from fat are called adipose stem cells and are an example of an adult stem cell. Adult stem cells are more limited in their ability to differentiate and are usually restricted to the lineages that they normally give rise to *in vivo*. Moreover, adult stem cells generally do not divide as quickly *in vitro* as embryonic stem cells, are often hard to obtain, and can usually not be passaged indefinitely. These factors make adult stem cells less attractive than ESC for use in toxicological assays. As a consequence, most toxicological studies involving stem cells have been done with ESC or cells differentiated from ESC. ESC were first isolated in 1981 from the inner cell mass of mouse blastocysts [7-8]. It was not until 1998 that similar lines of embryonic stem cells were derived from human blastocysts [10]. Embryonic stem cells are especially attractive candidates for toxicological testing as they are pluripotent, meaning they can develop into any cell type in an embryo and they can be passaged many times *in vitro*.

**Derivation of embryonic stem cells:** ESC are generally derived from whole blastocysts obtained from the reproductive tracts of mice or from spare blastocysts
offered for research purposes by patients undergoing in vitro fertilization. Trophoblast cells, which give rise to the placenta, are removed either by microdissection or immunodissection, and the inner cell mass is plated on a layer of mouse embryonic fibroblasts to generate a new cell line (Fig. 1.1). Because of their origin, embryonic stem cells per se are excellent models for the inner cell mass, while embryonic stem cells that have undergone differentiation model post-implantation development. In the pluripotent state, lines of hESC vary from each other, and some lines have a greater propensity to differentiate into certain lineages than others. It is important to consider this variation in toxicological work with this model. Multiple lines could be used to obtain the best overall information, or alternatively, generation of a line that represents the “gold standard” for toxicological testing may be possible.

There are several ways in which stem cells can be used to evaluate chemical toxicity (Fig. 1.2). In the first strategy, chemicals can be added directly to stem cells...
and their effects evaluated on endpoints such as maintenance of pluripotency, proliferation, apoptosis, survival, and growth. The endpoints can be evaluated using morphological or molecular criteria. For example, apoptotic blebbing and activation of caspase 3 are good morphological and molecular markers for apoptosis, respectively. Since embryonic stem cells come from the inner cell mass of blastocysts, this strategy models pre-implantation development. Alternatively, embryonic stem cells can be cultured using conditions that favor differentiation. This is often done by first forming embryoid bodies and then allowing the embryoid bodies to further differentiate into a particular lineage or differentiate spontaneously. Chemicals can be added either before or after embryoid body formation.
formation or at both times. Endpoints can include embryoid body formation, proliferation, or differentiation depending when the chemical is added. This strategy models the post-implantation embryo when differentiation of cells has begun. Finally, specific cell types, such as cardiomyocytes or hepatocytes, can be differentiated from embryonic stem cells and chemicals added to the differentiated cells. The differentiated cells can be fully or partially differentiated to enable comparison of fetal and adult counterparts. Endpoints can include apoptosis, survival, and retention of the differentiated state. This strategy is attractive as it allows mass production of large numbers of a specific cell type for testing (such cells may be difficult to obtain from a human source). Moreover, these cells can be fully or partially differentiated.

Induced pluripotent stem cells (iPSC) are the newest entry into the pluripotent stem cell arena\(^\text{13-14}\). These cells are usually derived from differentiated adult cells that have two to four genes ectopically expressed in culture. The first genes that were used to reprogram to a pluripotent state included Oct4, Sox-2, Klf4 and c-myc. In subsequent studies, it has been possible to reduce this list to just Oct4 and Sox2\(^\text{15}\). A small percentage of cells transfected with these genes reverts to a pluripotent state and becomes similar to embryonic stem cells. Although the possibility of using iPSC for toxicological studies is interesting, we need to have a better understanding of how close the iPSC model represents the pre and post implantation embryo and how faithfully cells differentiated from iPSC represent their \textit{in vivo} counterparts before they can be used with confidence in predictive toxicology and drug discovery.
Nevertheless, this avenue should certainly be explored and potentially represents an outstanding opportunity for future developments in toxicological testing and drug screening.

**Toxicological studies using mouse embryonic stem cells (mESC).**

**The mouse embryonic stem cell test (EST):** The mouse EST is an important assay that was developed in Germany in the 1990s. It is the only validated toxicological assay that uses stem cells and does not require animals (except for the initial derivation of the stem cell line). Its development was motivated by the movement in Europe to re-evaluate about 30,000 existing chemicals by the year 2015. To perform this re-evaluation and to carefully evaluate any newly released chemicals would require extensive financial and human resources and enormous numbers of laboratory animals. Movement toward highly predictive *in vitro* assays that can be used in conjunction with high throughput screening technology will be necessary to achieve this goal, as well as future evaluation of new chemicals. The EST is an evolving assay that may eventually be highly useful in solving the problem of screening large numbers of chemicals for toxicity. It continues to be modified and improved each year.

The EST is based on three endpoints (Fig. 1.3). For the first two, cytotoxicity of the test chemicals is measured using an MTT assay for both mESC (an embryonic cell type) and mouse 3T3 fibroblasts (a differentiated cell type). Cytotoxicity is evaluated using dose response experiments that give IC50s for both the 3T3 fibroblasts and mESC. The third endpoint is based on the capacity of D3 mESC to
differentiate into contracting cardiomyocytes. mESC are grown in the presence of leukemia inhibiting factor (LIF) that prevents differentiation. Cells are next allowed to aggregate into embryoid bodies in hanging droplets in the absence of LIF for 3 tissue culture dishes to allow outgrowth and differentiation of cardiomyocytes. By 10 days, some cells in the embryoid bodies will spontaneously differentiate into cardiomyocytes that are easily visualized microscopically without any further processing by their ability to contract spontaneously (a property not shared by other cell types). The differentiation assay is done at various doses, and an ID50 can be computed for each chemical. A biostatistical prediction model was developed based on the three EST endpoints and can place test chemicals in one of three categories of embryotoxicity (non-embryotoxic, weakly embryotoxic, strongly embryotoxic).
embryotoxic). The EST has been subjected to validation in an international study by the European Center for the Validation of Alternative Methods (ECVAM) in which 20 chemicals of known embryotoxicity were tested. Two other widely used in vitro assays (the micromass test on limb bud cells of mouse embryos and the post-implantation whole rat embryo assay) were compared to the EST in the validation study, and all performed favorably. The EST was as successful in predicting embryotoxicity of chemicals as the two other assays.

The main strengths of the mESC as it was originally developed are: (1) it has been validated with known embryotoxic chemicals and is currently the only in vitro assay based on stem cells that has been validated, (2) it is an excellent predictor of strongly embryotoxic compounds, (3) it can be done relatively easily in any lab set up for cell culture, and (4) it avoids the use of animals. The main disadvantages of the mouse EST are: (1) it is done with mouse cells that may not always accurately predict harm to humans, (2) as originally developed, it requires a long time to complete, e.g., about 10 days are required to do the differentiation phase of the assay, (3) it does not always correctly predict the toxicity of chemicals at the low end of the embryotoxicity spectrum, (4) it does not directly take into account maternal effects of the chemicals, (5) it does not measure toxicity of chemicals that may be deactivated or activated in vivo, and in its current form, embryotoxicants that are produced by metabolism will likely be missed (as an example of this problem, the EST missed cyclophosphamide, which is a strong teratogen that forms an active metabolite on first-pass metabolism), and (6) it is based on the
differentiation of only one mesodermal cell type, a possible limitation as other lineages may respond differently to a particular chemical. The above issues are being addressed as discussed below, and the EST continues to evolve into a stronger platform that will have more robust characteristics in the future.

**Improvements and additional evaluations of the mouse EST:** Since its introduction, the mouse EST has undergone a variety of enhancements. For example, the mechanics of testing have been improved by modifying the culture methods used to produce embryoid bodies. Since the standard operating procedure for making embryoid bodies for the mouse EST often did not yield the required 21 out of 24 contractile embryoid bodies needed to use the data, experiments often had to be discarded and repeated. To minimize this problem, Smedt et al (2008) developed a non-enzymatic method based on a buffered medium containing EDTA to obtain mES cells for making embryoid bodies and they standardized the hanging droplet culture. These modifications resulted in more tests having an acceptable number of differentiated embryoid bodies and produced embryoid bodies that were more uniform in size and quality and had stronger contractions, making evaluation of the differentiation endpoint more reliable.

Evaluating contractility in embryoid bodies requires considerable human labor. To accelerate counting of contractile embryoid bodies and remove human bias from the EST, Peters et al (2008) developed an automated method for evaluating the fraction of contractility from video data. While there was no statistically significant difference between data obtained using the automated video method vs. manual
assessment, there was considerable run to run variability in contraction, which may complicate the use of video parameters. While automating contractility would be helpful and could help move the EST to a medium throughput level, more evaluation will have to be done regarding the usefulness of the video approach in this application.

The original EST was developed using D3 mESC. Other lines have since been used with success. For example, a DBA/1lacj line derived at Pfizer gave results that mirrored those obtained in the original validation study by ECVAM. As was seen in the original study, the EST, when used with DBA/1lacj cells, predicted high risk compounds better than low risk counterparts. The study using the DBA/1lacj line also tested a number of receptor mediated pharmaceuticals with known in vivo toxicity. The EST did not predict the risk of these chemicals as well as it did the chemicals used to validate the assay, none of which were receptor-mediated drugs.

The DBA/1lacj study, in which numerous compounds including some that overlapped the original validation study, confirmed that the EST was a good, but not perfect, predictor of embryotoxicity and developmental toxicity. Clearly improvements in predictability can be made in the EST in the future to enhance its ability to predict receptor mediated drugs and chemicals that are weakly embryotoxic.

Two of the major drawbacks of the EST as it was first developed were the long time (10 days) required to reach the differentiation endpoint (contracting cardiomyocytes) and the reliance on only contracting cardiomyocytes, an endpoint
that could be misinterpreted, required considerable human time to evaluate, and
did not take into account possible effects on ectodermal and endodermal
derivatives. In an effort to improve the EST and reduce the time required to reach a
developmental endpoint, molecular markers have been introduced into the EST. A
new version of the EST, described as the FACS-EST, relies on fluorescence activated
cell sorting (FACS) to quantify two key cardiomyocyte proteins, sacromeric myosin
heavy chain and α-actinin. In a side by side comparison of 10 of the chemicals
used in the original validation study, the EST and FACS-EST compared very well in
their ability to predict embryotoxicity. Addition of FACS to the EST reduced the time
required to obtain differentiation data from 10 to 7 days and provided sound
quantitative molecular data as the differentiation endpoint. The reduction in time
required for the FACS-EST will make it more attractive to industries that need to
screen chemicals and drugs for potential embryotoxicity, but it still may not be fast
enough for medium and high throughput screening. One draw back to the FACS-EST
is that in moving to molecular markers of differentiation at earlier times, the
contraction endpoint is lost and with it valuable data on the physiology of the
cardiomyocytes.

A recent study investigated the possibility of reducing the time required to
access embryotoxicity with the EST even further by using transcriptomic techniques.
This study identified a set of 43 genes that are upregulated during the first 24
hours after plating embryoid bodies in differentiating conditions that favor
production of cardiomyocytes. The effect on gene expression was then examined in
embryoid bodies treated with monobutyl phthalate, a chemical that is known to produce birth defects, usually in structures of mesodermal origin. Monobutyl phthalate had the interesting effect of upregulating genes that are involved in pluripotency, proliferation, and non-mesodermal differentiation and downregulating the gene set expressed during cardiomyocyte differentiation. These data suggest that transcriptome analysis could be used to detect the earliest changes in cardiomyocyte development and that as early as 24 hours after plating embryoid bodies on low attachment plates, marker genes are expressed that could serve this purpose. While additional work would need to be done to validate this approach, shifting to transcriptome analysis could reduce the time to evaluate cardiomyocyte differentiation to 4 days rather than 7 days, which would represent a significant improvement in the application of this assay. However, the time and cost to analyze gene expression vs. contracting cardiomyocytes or FACS analysis of cardiomyocytes would need to be factored into this.

The evaluation of contracting cardiomyocytes is a suitable straightforward morphological assay for mesodermal differentiation. However, the EST could be improved by complementing cardiomyocyte contraction with molecular markers and by extending the EST to ectodermal and endodermal lineages, and to mesodermal derivatives other than cardiomyocytes. In 2004, a modified version of the EST was introduced in which expression levels of markers for osteogenic, chondrogenic, neural, and cardiac differentiation were quantified after exposure to six toxicants that were non-teratogenic (penicillin), moderately teratogenic
(diphenylhydantion, valproic acid, thalidomide), and strongly teratogenic (5-fluorouracil and retinoic acid)\textsuperscript{27}. The molecular multiple endpoint EST (mme-EST) correctly classified each test chemical and additionally showed that some chemicals produced interesting differences in their effects on the three lineages. For example, retinoic acid inhibited osteogenic and chondrogenic differentiation, but not neural differentiation. In a related study that examined molecular markers for cardiac and bone differentiation in the presence of methotrexate, the expression of bone markers by mESC decreased, while cardiac marker expression remained at control levels\textsuperscript{26}. These results are in agreement with known effects of methotrexate \textit{in vivo}. Both the mme-EST and the methotrexate studies demonstrate the importance of using multiple endpoints that take into account different lineage markers as lineage derivatives clearly differ in their response to the same chemical.

van Dartel et al (2009) have shown that EST results can be affected by inhibition of both cell proliferation and differentiation, and they propose considering both of these parameters in the prediction model\textsuperscript{28}. By exposing embryoid bodies from day 0 - day 10, the processes of proliferation and differentiation are not studied separately from each other. However, exposing cells from day 3 onward would give a better read-out for the effects of test compounds on differentiation. This suggestion has been recently made, and it is too early to know if it will be widely adopted.

It would also be highly desirable to increase the output of the EST. Progress toward this goal has been made recently by adapting the EST to 96 well low
attachment dishes. Twelve chemicals with known embryotoxicity were compared using the EST endpoints. This method predicted embryotoxicity in good agreement with the original EST and indicates that movement to a 96 well plate format is feasible and could enable large sets of data to be collected. The assay, however, is not fundamentally geared to high throughput as the time to reach the differentiation endpoint in this study was still 10 days. Combination of the 96 well format with an earlier molecular readout would help move the EST to a higher throughput mode.

ESC, which are pluripotent, provide an enormous benefit in that any cell type can potentially be differentiated from them. The EST has been expanded recently to include differentiation of endothelial cells. An efficient method was first developed to differentiate endothelial cells in embryoid bodies created in hanging droplets, and an assay based on the EST was then developed, except that the differentiation of endothelial cells, not cardiomyocytes, was monitored. Differentiation of endothelial cells was accessed by examining expression of PECAM-1 and VE-cadherin with real time PCR. Six known embryotoxicants (all-trans-retinoic acid, 5-fluorouracil, diphenylhydantion, valproic acid, saccharin, and penicillin G) were tested in this assay which correctly classified the toxicity of each. With further work, this method could be developed to include a predictive model that would be useful for screening chemicals with unknown effects on embryos. It is probable that other differentiation endpoints will be added to the basic EST as it evolves. It would be helpful to expand this assay to also include differentiation of endodermal and ectodermal derivatives.
Finally, the EST could be combined with in silico assessment of risk to determine the in vivo effect levels for developmental toxicants. Some preliminary work has been done in this area, and the in vivo effect levels were correctly predicted for four of five tested toxicants. If further refined, such an approach may help to further reduce the need for animals in toxicological assessment studies.

**Use of the mouse EST to evaluate toxicants that were not tested during its original validation.** The mouse EST has been employed by a number of laboratories to evaluate toxicity of compounds not originally tested during validation. Several examples will be given.

Since many test chemicals need to be dissolved in solvents for testing, Adler et al (2006) have evaluated the effects of dimethylsulfoxide (DMSO) and ethanol on the ability of mESC to maintain pluripotency. These two solvents are widely used to dissolve chemicals used in the EST. A reporter line of mESC that had been transfected with the mTert promoter coupled with the GFP gene was used. Analysis of solvent treated cells by flow cytometry and evaluation of Oct4 expression by semiquantitative RT-PCR revealed that DMSO could induce differentiation (loss of mTert and Oct4 expression). They recommended that these solvents be used in doses no higher than 0.1% for DMSO and 0.25% for ethanol to avoid induction effects.

To determine if the EST could accurately evaluate chemicals within a distinct chemical class, the toxicity of glycol ether alkoxy acid metabolites was compared using the EST and data obtained from in vivo studies. At doses that were not
cytotoxic, all tested compounds showed a dose dependent inhibition of cardiomyocyte differentiation. The hierarchy of potency from most to least potent was: methoxyacetic acid, ethoxyacetic acid, butoxyacetic acid, phenoxyacetic acid. The data obtained with the EST for this group of chemicals were in good agreement with *in vivo* data indicating that the EST can be used to predict toxicity of chemical groups of compounds. This study also found that variation between labs in performance of the EST was within acceptable limits.

Fluoxetine is an antidepressant that is often prescribed for women to treat mood disorders during pregnancy and lactation. Initial clinical trials and animal studies did not detect any adverse effects of fluoxetine on adults or prenatal development. However recent reports of increased incidence of several birth defects prompted a study of fluoxetine’s effects using the EST, which showed fluoxetine adversely affected cell viability and differentiation of mESC into cardiomyocytes. It was further found, by examining markers, that fluoxetine impaired mesodermal differentiation. These data in combination with recent reports of congenital defects in the offspring of fluoxetine users suggest caution in taking this drug during pregnancy and show that further study into its effect on developing young is needed. This is an interesting example of the mouse EST detecting toxicity of a chemical that was not found to be toxic in earlier work.

The only strongly embryotoxic chemical to be incorrectly classified as non-embryotoxic in the original EST validation study was methylmercury. This led others to hypothesize that the EST may not be able to correctly classify heavy
metals. When cadmium and arsenic compounds that were known to be embryotoxic in vivo in mice were tested using the EST, the test failed to identify one cadmium and two arsenic compounds as embryotoxic\(^{36}\). When all tested heavy metals are considered together, the EST failed to correctly classify four out of seven. These data indicate the need for modifications or improvements to the EST so as not to miss important toxicants that have serious effects on embryos.

In an interesting application, the toxicity of eleven metals used in dental alloys were evaluated in the EST\(^{37}\). Mercury and chromium were classified by the EST as strongly embryotoxic, in agreement with other studies on these two metals \(^{38-39}\). Antimony, tin, and vanadium ions were weakly embryotoxic, while silver, cobalt, copper, nickel, palladium, and zinc were not embryotoxic. However, as discussed above, the ability of the EST to identify toxic metals has been called into question\(^{36}\), and the negative data in this study may need re-evaluation by an alternative method.

**Studies that have tested toxicity using mESC without using the EST:** mESC have been used by a number of labs to evaluate toxicity of environmental chemicals without using the EST. For example, the effect of arsenic on the expression of selenoproteins in mESC was studied with the CGR8 cell line\(^{40}\). Selenoproteins play important roles in humans in antioxidation, redox regulation, and detoxification. Arsenic was found to up-regulate the expression of selenoproteins associated with antioxidation, while downregulating selenoprotein H and some of the
selenoproteins located in the endoplasmic reticulum. Selenium was able to restore expression of the downregulated proteins in mESC.

Potassium dichromate [Cr(IV)], which is commonly used in laboratories and industry, is a widespread environmental toxicant. Its mechanism of action has been studied in depth using mESC to evaluate the signal transduction pathways activated by [Cr(IV)]\textsuperscript{41}. [Cr(IV)] was shown to activate both p38 and JNK, but not ERK via MAP2K4 and MAP2K7. Phosphorylation of p38 produced cytotoxic effects in mESC, while activation of JNK inhibited cytotoxicity as well as differentiation of mESC into cardiomyocytes. This very thorough study approached the actions of [Cr(IV)] at multiple levels in the mESC model and provides a more complete understanding of the adverse effects of [Cr(IV)] on embryonic cells.

mESC stably transfected with the gene for green fluorescent protein under the regulation of the cardiac α-myosin heavy chain promoter were used to test various compounds in restorative dental materials \textsuperscript{42}. This study also measured cytotoxicity of test compounds on mESC using the MTT assay. Chemicals were considered embryotoxic if they decreased expression of GFP without causing cytotoxicity. This assay was therefore similar to the EST except that transfected cells were used to monitor cardiac differentiation rather than contraction of cardiomyocytes and cytotoxicity was not compared to 3T3 cells. Prior to testing dental compounds, this assay (R.E. Tox assay) was validated using 20 reference chemicals and found to accurately predict embryotoxicity. When dental restoration compounds were then tested, three compounds were not cytotoxic and did not affect differentiation, while
the remaining compounds produced various effects on both cytotoxicity and
differentiation with methacrylic acid significantly stimulating differentiation at non-
cytotoxic levels. This study demonstrates the potential to use GFP reporter cell lines
in a modified version of the EST.

Paraquat, a highly toxic quick acting herbicide used in agriculture, was tested
extensively using mES cells with a spectrum of biological assays. When stem cells
were examined after 24 hours of exposure, paraquat was found to stall cell
proliferation, increase reactive oxygen species, and increase apoptosis and necrosis.
These adverse effects could be prevented or reduced by inclusion of vitamin C in the
culture medium containing paraquat. The data in this study are important in
suggesting that paraquat may be dangerous to young embryos at concentrations
that have not previously been considered harmful.

Valproic acid, a chemical known to cause defects in the heart and nervous
system, was tested using mESC and cardiomyocyte differentiation, although the
standard operating procedure for the EST was not used in this study. Valproic acid
inhibited growth of embryoid bodies at levels that were not cytotoxic, decreased the
number of contracting embryoid bodies, and decreased the area of contraction in
plated embryoid bodies. These effects coincided with increased levels of reactive
oxygen species in valproic acid treated embryoid bodies. The effects of valproic acid
on cardiomyocyte differentiation could be reversed by vitamin E treatment. In
related studies, the anticonvulsants carbamazepine and valproic acid were studied
using differentiation of contracting cardiomyocytes and the expression of marker
genes for differentiation as endpoints. In all assays including cytotoxicity, inhibition of expression of endodermal and mesodermal lineage markers, and induction neuronal differentiation, valproic acid was more potent than carbamazepine. These results were in good agreement with known in vivo data for these two anticonvulsants and demonstrate the usefulness of mESC to study and compare toxicity of therapeutic drugs.

Studies using differentiated mouse embryonic stem cells: In addition to the EST, numerous studies have been done in the past 5 years using mouse embryonic stem cells in other experimental designs. One strategy, for example, is to differentiate specific types of cells from ESC and use the differentiated cells in toxicological testing. This would be a way, in principle, to obtain any cell type in large enough numbers to conduct toxicological experiments. Moreover, different lineages could be examined which is ultimately important since cardiomyocytes may not be responsive to all toxicants. Examples of some of this work will be presented. Most studies have allowed ES cells to differentiate into another type of cell, and then evaluated the toxicity of test chemicals.

While the EST relies on differentiation of cardiomyocytes, some labs have explored chemical toxicity using neurons differentiated from ESC. The idea of using neurons is attractive since the central nervous system is very sensitive to environmental chemicals throughout the prenatal period. In the original EST validation study, the EST failed to detect methylmercury as embryotoxic. This chemical produces malformations in brain development indicating the nervous
system, not the heart, may be its major target. Stummann et al (2007) examined the effect of methylmercury on the differentiation of mESC into neuronal-like cells and found downregulation of expression of Mtap2, a marker for neuronal differentiation. However, several other neuronal markers were not affected by methylmercury indicating that multiple markers need to be used when gene expression is the endpoint. In a subsequent study using hESC, methylmercury inhibited differentiation of ESC into a neuronal precursor-like cell but was less effective at inhibiting maturation of the precursor cells into neuron-like cells.

With hESC, the expression level of all markers except nestin decreased during methylmercury exposure, in contrast to the mESC experiment in which only Mtap2 decreased. This illustrates that different markers need to be used when evaluating toxicity with different species and further shows that results were highly dependent upon the stage of neuronal differentiation that was examined.

Endothelial cells differentiated from mES cells have also been tested with 5-fluorouracil, a chemical known to inhibit vasculogenesis. Endothelial cells derived from mES cells were somewhat more sensitive to 5-fluorouracil than adult mouse endothelial cells in a growth assay, supporting the general idea that embryonic cells are more sensitive than adult cells to toxicants. This observation which is supported by diverse data is important as testing the most sensitive stage in the life cycle should arguably be the benchmark for toxicologically studies. Further work showed that the viability of endothelial cells derived from ES cells is significantly reduced by 24 hours of exposure to 5-fluorouracil (10 M), an effect that
was reduced by simultaneous exposure to probucol (50 M) and that 5-fluorouracil also decreased proliferation and differentiation of endothelial cells and induced the G1/S phase arrest in the cell cycle\(^{51}\). These authors suggest that endothelial cells differentiated from mES cells could be a valuable model for screening for toxicity in new chemical compounds. Certainly interference with vasculogenesis or angiogenesis would be a significant problem prenatally and postnatally, as these are times when new vessel development is essential. Adaptation of this strategy to hESC would be beneficial.

Obtaining pure populations of differentiated cells for toxicological work is an area that needs attention. While controlled differentiation methods are continually improving and yields of specific cell types continue to increase, other strategies for obtaining pure cell types for assay development have also been investigated. For example, Chaudhary et al (2006) have used laser microdissection and pressure catapulting (LMPC) to isolate contracting cardiomyocytes from differentiating embryoid bodies\(^{52}\). These isolates, which expressed cardiac markers and exhibited the functional characteristics of cardiomyocytes, can be transferred to 96 well plates for further study. They suggest that this method could be used to isolate homogeneous ESC-derived cell types for heterogeneous populations of differentiating cells.

Other novel strategies for measuring cytotoxicity using mES cells have been developed. For example, Calabro et al (2008) have measured transepithelial electrical resistance in monolayers of mES cells that were grown using
differentiation conditions, and found that resistance decreased proportionally to increases in cytotoxicity. This model could be developed into a valuable assay for measuring cytotoxicity with embryonic cells, although a number of cytotoxicity assays currently exist.

**Human embryonic stem cells in toxicological testing. Examples of studies that have been done:** While usually thought of in the context of regenerative medicine, hESC also provide one of the best opportunities available for developing assays to assess toxicity of environmental chemicals, and thereby helping to prevent disease. hESC can be used to model the earliest stages of human development and can be differentiated into cells with characteristics of those found in embryos. Experiments can be designed to model undifferentiated hESC, differentiating hESC, or hESC that have differentiated into a progenitor or mature cell. Specific types of differentiated cells, such as hepatocytes and cardiomyocytes, would be especially valuable in testing toxicants, but in principle any type of cell could be produced and studied using hESC. So while hESC have great potential to treat and cure regenerative diseases, they also have equally great potential to prevent disease by identifying dangerous environmental chemicals and drugs before they cause harm.

In spite of the fact that hESC represent one of our best opportunities to develop methods for screening chemicals that may be toxic to humans, relatively little work has been done with them in this context. A human counterpart to the mouse EST has not yet been developed. This is in part because hESC are more difficult to work with than mouse cells and present certain unique challenges that must be overcome.
before well accepted assays are developed with them. For example, hESC grow slower than those from the mouse, they tend to clump, and they are more difficult to grow as single cells, a fact that could be important in assays involving cell quantification and homogenous distribution for cells in treatment groups. Also it has been difficult to efficiently and consistently differentiate hESC into contractile cardiomyocytes, but recent improvements in this technology\textsuperscript{54-55} could facilitate development of a human EST patterned after the mouse model.

One recent paper developed the framework for a human Embryonic Stem Cell test\textsuperscript{56}. Two embryotoxicants, retinoic acid and 5-fluorouracil, were used to develop a cytotoxicity assay comparing the sensitivity of hESC and human embryonic lung fibroblasts. In addition, quantitative RT-PCR was used to identify potential marker genes that could be used to monitor cardiac differentiation. The most useful markers identified in this study were brachyury and GATA-4 for cardiac differentiation and the late cardiac gene TNNT2, which was expressed between days 10 and 18. This study clearly shows that progress is being made toward the development of a human EST, but we are still far from completing this goal.

A second interesting study has evaluated the potential of hESC to provide information on toxic chemicals using two non-embryotoxic, two weakly embryotoxic, and two strongly embryotoxic chemicals\textsuperscript{57}. Cytotoxic indices were determined using cells representing the embryo (hESC), fetus (human embryoid bodies) and adults (human foreskin fibroblasts). In addition, the effects of each chemical on differentiation were monitored using lineage specific markers. Both
embryoid bodies and hESC were more sensitive to chemical treatment than the foreskin fibroblasts in the survival assay. In the gene expression assays, non-embryotoxic chemicals were without effect (penicillin) or only effective at high doses (saccharin), the weakly embryotoxic chemical, indomethacin, downregulated endodermal markers, while busulfan which is strongly embryotoxic downregulated most of the markers. This study further correlated effective doses of each chemical with doses that were found in the serum of patients receiving these drugs and found that several were within the range present in human patients. These data showed that hESC and human embryoid bodies can be used to monitor toxicity of drugs and environmental chemicals; however, much further work is required to fully develop the potential of human stem cells for this purpose.

hESC were used recently to evaluate the ability of nonylphenol and octophenyl, two environmental contaminates that disrupt the reproductive and endocrine systems, to induce apoptosis\textsuperscript{58}. Using a variety of methods, this study found that these test chemicals did induce apoptosis in hESC via a Fas-Fas ligand pathway in which caspase 8 and 3 activation increased following exposure to the test chemicals. hESC were also differentiated into neural progenitor cells which were found to be more sensitive to the two test chemicals than the hESC. This may be due to the presence in the hESC of ABC transporters which are able to protect these cells against stress.

Additional work supports the idea that differentiated or differentiating embryonic cells may sometimes be more sensitive to toxicants than hESC. In a
survivability assay (MTT), hESC were more resistant to oxidative stress than fibroblasts that had differentiated from hESC. Survivability was not enhanced for either cell type by heat-shock pretreatment or by preconditioning with low levels of oxidative stress. A similar finding has been reported for mESC which were more resistant to oxidative stress than embryonic fibroblasts. In the mouse study, several antioxidant-related genes were down regulated as mESC differentiated into embryoid bodies. hESC and mESC thus appear to be more resistant to oxidative stress than newly differentiated cells, and their use in toxicological assays would need to consider this point.

In an interesting use of hESC to access arsenic toxicity, hESC were exposed to arsenic, then the ability of an arsenic antidote (monoisoamyl dimercaptosuccinic acid) to rescue the cells was evaluated using cytotoxicity and gene expression endpoints. In addition, rats were treated with both arsenic and the antidote, and litter size and developmental defects were examined. The hESC assays were able to detect damage done by arsenic and reversal of damage by the antidote, and the in vitro observations with stem cells correlated well with the in vivo data on arsenic induced damage.

**Differentiation of hESC and Toxicity Testing:** Another strategy for using hESC is to first differentiate them into a specific cell type of interest then subject the differentiated cells to toxicological testing. Hepatocytes and cardiomyocytes could be useful for tools for this purpose, while other cells may also be highly interesting. For example, should it become possible to differentiate oocytes from hESC, they
could be used to assess sensitivity of the germline to environment toxicants. Effects of toxicants on sperm and oocytes are important as they are fundamental to reproduction of our species and detection of damage may not occur for many years after exposure.

In a study on fibroblasts spontaneously differentiated from hESC, the cytotoxicity of mitomycin was measured using the MTT assay\textsuperscript{62}. The differentiated population was heterogeneous, but this group argues that this will be satisfactory as the results they obtained were reproducible. This could be an important point as obtaining highly purified differentiated cells is not always easy and may not fit well into future high throughput operations. Nevertheless, improvements in directed differentiation are continually being made, and in the long term, the need to work with heterogeneous population of differentiated cells should not be an issue.

Because the nervous system is very sensitive during all phases of prenatal life, finding ways to identify toxicants that affect its development is very important. Recently neurospheres derived from hESC have been used to evaluate neurotoxicity in a three dimensional model\textsuperscript{63}. In this study, neurospheres were tested with teratogens classified as strong (hydroxurea), weak (valproic acid and lithium), and non-teratogenic (acrylamide). The endpoints included cytotoxicity and neuronal protein marker expression. Valproic acid, which was the most effective chemical tested, reduced viability and decreased expression of neuronal markers, in agreement with studies using the mme-EST with mouse stem cells\textsuperscript{27}. However not all applications of valproic acid have reached the same conclusion. In differentiation
tests using mESC, valproic acid was found to promote neuronal differentiation, not inhibit it.\textsuperscript{46,64-65} This discrepancy may be due to species differences or to the different methods that were used to induce neuronal differentiation, a factor that will need to be considered carefully when developing a general assay using stem cells for toxicological testing. In contrast to valproic acid, lithium did not effect viability or marker expression in the neurosphere study.\textsuperscript{63} Hydroxurea and acrylamide reduced viability but did not alter marker expression. Thus, the neurospheres were useful in indentifying neurotoxicants, but the hierarchy of their potency obtained with the neurosphere test was not an exact match for their known in vivo toxicity. As often the case, chemicals at the low end of the toxicity spectrum are the most difficult to classify. In this case acrylamide was weakly embryotoxic rather than non-embryotoxic. However, the fact that acrylamide did have an effect on viability may be important. In vivo assays that rely largely on anatomical changes and non-molecular endpoints may not detect subtle changes in cells that are revealed by in vitro assays. This begs the question - do we need to be more careful with chemicals that are classified as non-embryotoxic - we could be missing information that our traditional in vivo tests do not reveal.

Differentiated neurons are also of interest and can be obtained from hESC. It is difficult to grow and maintain neurons from the adult brain, but hESC provide a means to create an unlimited supply of differentiated neurons for toxicological and neurological studies. In a recent study, hESC were differentiated into dopaminergic derived neurons and treated with 1-methyl-4- phenylpyridium (MPP+) , which
produces features of Parkinson's disease in humans. MPP+ caused an increase in reactive oxygen species in treated neurons that was accompanied by an increase in apoptosis. Neurons could be rescued from the effects of MPP+ by treatment with glial cell line derived neurotrophic factor. This study demonstrates the potential usefulness of hESC in differentiation of cells that may otherwise be difficult to obtain and grown in culture for studies of toxicity.

Use of ESC to evaluate the toxicity of cigarette smoke on prenatal development. Cigarette smoke is a complex mixture of chemicals, many of which are known toxicants and carcinogens. Nicotine, the major bioactive chemical in cigarette smoke, is addictive. Numerous epidemiological studies have shown that in utero exposure to cigarette smoke decreases birth weight significantly and may produce other unwanted effects such as increased risk for placenta abruptio, stillbirth, ectopic pregnancy, preterm birth, and SIDS. The epidemiological data are supported by animal studies, many of which have been done using in vitro technology. Of additional concern are the recent findings that children exposed to smoke in utero have cognitive and learning problems after birth. While it is not feasible to experimentally test the full range of effects of smoke on actual human embryos, it is possible to experimentally evaluate smoke’s effect on human prenatal development by using ESC as an in vitro model for human embryos. Cigarette smoke can be studied both as mainstream smoke (MS), which is inhaled in each puff by active smokers, and as sidestream smoke (SS), which is the smoke burning off the tip end of a cigarette. In addition, whole smoke or individual components in smoke
can be studied using ESC-based assays. Thus ESC provide a model that can be used to gather information on how the earliest stages of human prenatal development are influenced by exposure to smoke. This is an important point as these stages are usually the most sensitive to environmental chemicals and are likely to be the most severely impacted by smoke.

The effects of MS and SS cigarette smoke from both conventional and harm reduction cigarettes have been studied using mESC\textsuperscript{79}. Both MS and SS smoke inhibited attachment, survival, and proliferation of mESC dose dependently. Pretreatment of cells with smoke solutions, followed by washing and plating in control medium also reduced attachment of treated cells. Moreover, when mESC were first plated then treated with smoke, they detached from the substrate in a dose dependent manner. In side by side comparisons of traditional and harm reduction cigarettes (which are often claimed to be less toxic), smoke from the harm reduction brands was unexpectedly found to be more potent than smoke from the traditional brand when assayed with mESC. In addition, SS smoke was consistently more toxic than MS smoke from all brands. To verify that embryos respond similarly to smoke solutions, mouse pre-implantation embryos were recovered from oviducts and treated \textit{in vitro} with MS and SS smoke solutions. In treated groups, blastomeres were often lysed, and caspases 3 & 7 were activated indicating the occurrence of apoptosis. These observations are important in calling attention to the sensitivity of the earliest stages of development to toxicants in both MS and SS and further
highlight the need to better understand smoke from brands that are purported to reduce harm.

In a subsequent study, hESC were grown on Matrigel, detached using Accutase, then replated as single cells in control medium or medium containing a non-cytotoxic dose of SS smoke from a conventional brand (Marlboro Red), and followed using time lapse video in a BioStation IM. The percentage of attached cells over a 90 minute period was significantly higher in the control group than in the group treated with SS smoke solution (Fig. 1.4). These data with hESC, while preliminary, are in agreement with the mouse study\textsuperscript{79} and again show that smoke treatment impairs attachment to and spreading of embryonic cells on an extracellular matrix. Attachment of cells to extracellular matrices is important in all phases of embryogenesis, and factors that adversely affect attachment would be expected to impair normal development.

In a related study using hESC, nicotine, the major bioactive component of cigarette smoke, dose dependently inhibited cell attachment to Matrigel, an effect that was reversed by tubocurarine, a nicotine antagonist\textsuperscript{80}. The doses of nicotine

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_1.4}
\caption{SS smoke from Marlboro Red cigarettes significantly inhibited attachment and spreading of H9 hESC on Matrigel relative to control cells that were untreated. A non-cytotoxic dose (0.1PE) of smoke solution was used in this experiment. Multiple fields (N = 5) were monitored and assayed in both groups. Data were analyzed from time lapse videos collected with a BioStation IM.}
\end{figure}
that were effective (1.8 and 3.7 μM) were below the dose (6 μM) reported in the cervical mucus of the female reproductive tract. The hESC responded morphologically to nicotine by undergoing extensive vesiculation which was reversible upon washout. Nicotine was also found to increase the percentage of apoptotic cells above control levels in both unattached and attached cells. Overall, nicotine affected a number of endpoints, and the reversibility of some of these effects by tubocurarine suggests that hESC have a receptor for nicotine. While these data show that nicotine is one agent in tobacco smoke that can adversely affect hESC, it is probable given the complexity of smoke, which contains well over 4,000 chemicals, that other chemicals also produce adverse effects on hESC. The effect of nicotine on expression of pluripotency markers in embryonic stem cells is not yet clear. In mESC, nicotine doses that bracketed those found in human smokers were found using quantitative RT-PCR to increase expression of Oct-4 and Rex-1, two genes associated with pluripotency. This effect was prevented by tubocurarine, a nicotinic acetylcholine receptor antagonist. In contrast, hESC pluripotency markers appeared to decrease when cells were exposed to nicotine. This discrepancy could be due to species differences (mESC vs. hESC) or culture conditions (mESC experiments were done on feeder layers, while those with hESC were done directly on Matrigel).

Preliminary data on embryoid bodies derived from D3 mESC further demonstrate adverse effects of cigarette smoke on differentiation. In a cytotoxicity assay using trypan blue, mESC were treated with 0.1 or 1.0 puff equivalents (PE) of
MS or SS smoke from a harm reduction brand during formation into embryoid bodies. One PE equals the smoke in one puff that dissolves in 1 ml of medium. While neither dose of MS smoke affected formation and growth of embryoid bodies, the high dose of SS smoke (1.0 PE) inhibited aggregation of mESC into embryoid bodies, and most cells stained with trypan blue, indicating cell death had occurred (Fig. 1.5). In a follow-up experiment using RT-PCR to evaluate gene expression, mouse embryoid bodies, which were formed and incubated in LIF containing medium, were exposed to 0.1 PE of MS or SS smoke solution from a harm reduction brand of cigarette (Advance Lights) for 14 days to determine if smoke treatment affected differentiation of the three germ layer and/or maintenance of pluripotency (Fig. 1.6). Rex1, which is usually the first pluripotency marker to decrease as differentiation begins, was downregulated in cells treated with MS, but
not SS smoke solution suggesting that MS smoke accelerated differentiation within the embryoid body. Other pluripotency markers remained high, probably because the medium contained LIF. Analysis of additional gene expression showed up-regulation of the endodermal (GATA-4 and α-fetoprotein) and mesodermal (T-gene) markers by both MS and SS smoke, while no effect was observed on the expression of the ectodermal markers (nestin and neuroD). The data showed that cigarette smoke exposure can alter the timing of gene expression in the early stages of development, a point that should be studied further.

**Potential Pitfalls with Stem Cells:** While stem cells provide one of the best opportunities to develop methods for toxicological testing, it is important to be aware of pitfalls connected with their use. The substrate used for ESC culture can affect the outcome of toxicological tests. For example, Matrigel outperformed gelatin...
in assays that measured maintenance of pluripotency, response of mESC to sodium arsenite, and percentage of cells that developed into cardiomyocytes. Stem cells in culture may also undergo changes with repeated passaging on Matrigel. For example, mESC when repeatedly passaged in the presence of high LIF on Matrigel showed variable responses to caspase 3 activation by sodium arsenite. Also passaging can lead to aneuploidy or more subtle chromosomal translocations or deletions, which may affect the outcome of toxicological tests. Stem cells are also subject to contamination by bacteria, fungi, and Mycoplasma which can cause setbacks in performing assays, and are especially devastating when differentiating cells over long periods of time.

Some data clearly show that different labs can obtain similar results when using the EST; however, when standardized tests are not used, results can be variable was seen with valproic acid studies. Standardization of differentiation protocols will be important to obtain meaningful data among different labs. It is also clear from studies that have already been done that the time of exposure can effect the interpretation of the results. Chemicals will generally act at a specific time in development, and if that time is not included in the assay, the toxicity of the tested chemical will be missed. We have also seen that not all markers are affected by a particular treatment. In assays that rely on differentiation markers, clearly more than one marker for a particular lineage needs to be used, and ideally derivatives of the three germ layers and germ cells would be studied.

**Future improvements:** The potential of hESC to be used in assays that predict
toxicity of environmental chemicals and drugs is enormous. Investment in the development of new toxicological assays based on hESC could pay off enormously and help keep the planet healthy by preventing unwanted disease. hESC can meet an urgent need to develop new methods for toxicological testing that are faster, high throughput, cost effective, and based on human cells. Unfortunately, the value of hESC in toxicology is often overshadowed by their therapeutic potential to treat degenerative disease. In this closing section, some of the improvements and areas for future research will be considered.

Improved methods to culture and differentiate embryonic stem cells are developing rapidly and will enhance the options available for toxicological testing. More defined media have already been introduced for both mouse and human ESC 85-86, and it is likely that culture media will continue to improve. Interesting new hydrogels are becoming available that will provide three dimensional scaffolds for mimicking the in vitro environment more precisely than two dimensional matrices often used today 87. Artificial hydrogels can also be prepared free of undefined growth factors that could influence the outcome of toxicological testing. The mouse EST is based on differentiating cardiomyocytes. It is clear from the literature that it is important to examine effects of chemicals on ectodermal and endodermal lineages as well. Adding new differentiation protocols to assay development will help improve their predictivity.

Pure cultures of differentiated cells, such as hepatocytes and cardiomyocytes, will be extremely valuable in the future in both drug and chemical testing.
Hepatocyte-like cells differentiated from hESC show many characteristics of true hepatocytes, such as glutathione transferase activity \(^{88}\), which is encouraging news. As the research effort in regenerative medicine evolves, improved protocols for differentiating stem cells into specific cell types will improve, and toxicologists can take advantage of the availability of this resource and integrate specific differentiated cells into platforms to screen for potential benefits or harm. Validation of new assays is important. Although the mouse EST has been successfully validated, no assays based on hESC have yet been subjected to validation. While time consuming, validated assays will be important in the future of predictive toxicology. Validation can come only after a viable assay(s) has been developed and preliminarily tested. While validation will be a project for the future, planning for it can begin now.

Embryonic stem cells have an important advantage over adult cells in that they enable studies to be done on cells that represent stages in prenatal development. Because these stages are generally the most sensitive in our life cycle, it is often argued that the embryonic and fetal stages are the ones that should be used when assessing the risk of specific chemicals to humans\(^2\). As an additional bonus, stem cells provide a means to look at various stages of prenatal development including pre-implantation embryonic cells, post-implantation differentiating cells, and fully differentiated cells representative of fetal stages.

Consideration needs to be given to the metabolism of test chemicals which can inactivate or active toxicants in any assays that are used for human testing. The EST
does not take into account maternal factors or metabolism of the test chemical and as originally developed will probably not be a good predictor of toxicants that are affected by metabolism. Combining the EST or hESC based assays with in vitro liver extracts, such as S9, may help improve their ability to identify the full range of chemicals that are embryotoxic.

Improvements in reducing the time required to collect data are important. Methods that enable medium or high throughput screens with ESC are needed and are in development\(^8^9\). Other strategies such as signalomics, which involves high throughput screens to reveal simultaneous alterations in signal transduction cascades in response to a test chemical, could be valuable for future development of in vitro assays for drug and chemical testing\(^9^0\)-\(^9^1\). With high throughput methods will come a need for sufficient numbers of both hESC and specific cell types differentiated from hESC. The ability to scale up production of hESC for use in high throughput assays will be an important adjunct to the development of the assays per se.

By moving to a human cell based platform, the potential to develop better, more accurate tools for predicting toxicological outcomes should greatly improve. As recently pointed out by Greaves et al (2004), the dog, which is rarely used in toxicological studies, is a better predictor of human toxicities than the primate, rat or mouse, and even the dog misses about 37% of the chemicals toxic to humans\(^9^2\). Because animal studies are expensive, the transition to human stem cell based assays could be both cost effective and provide better predicative data than the
currently used animal models.

The future of predictive toxicology has never looked better. With the potential to use hESC in the development of new assays and to be able to study specific cell types as well as cells that model young embryos, we can expect in the future to have vastly improved methods for screening chemicals before human exposure occurs and for screening potential drugs that may alleviate human disease.

Addendum to chapter 1:

Chapter 1 reviews the literature pertaining to my Ph.D. dissertation entitled “Using Embryonic Stem Cells as a Novel Model to Compare the Toxicological Effects of Harm Reduction and Conventional Cigarette Smoke on Early Embryo Development”. The proceeding chapter has been accepted by Current Topics in Medicinal Chemistry and is in press for publication.

My dissertation deals specifically with the effects of MS and SS cigarette smoke on pre-implantation development. Cigarette smoke, composed of more than 4,000 chemicals (EPA, 1992), is made up of two classes of smoke, MS smoke, smoke actively inhaled by the smoker, and SS smoke, smoke burning off of the tip of the cigarette (EPA, 1992). Cigarette smoke is the leading cause of death in the United States, and can also induce a number of health problems such as cancer, heart disease, lung disease, reproductive impairment, and early childhood defects (CDC, 2000-2004).
As more studies have shown that smoking is extremely detrimental to human health, smokers and tobacco product manufacturers are advocating the use of harm reduction cigarettes. Harm reduction cigarettes, also known as “light” cigarettes or PREP (Potentially Reduced Exposure Products), are marketed as safer than conventional brands with reduced amounts of tar, nicotine, and other carcinogenic toxicants. Harm reduction cigarettes are manufactured in different ways: (1) reduction of toxicants with the addition of advanced filters (i.e. “trionic” filters), (2) genetic modification of tobacco plants to have lower nicotine concentrations, and (3) palladium cured tobacco leaves to reduce carcinogenic nitrosamines during burning. Although harm reduction cigarettes may contain lower tar and nicotine concentrations, light cigarette smokers, who are addicted to nicotine, experience more frequent nicotine withdrawals. As a result, the number of cigarettes smoked increases, and smokers generally take bigger puffs thus inhaling more non-nicotine smoke toxic components, which could potentially cause more harm than conventional brand cigarettes (Hatsukami, et al., 2004). Although a number of reports have shown that conventional brand cigarette smoke causes cardiac, respiratory, and reproductive impairments, little is known about the effects of harm reduction smoke on pre-implantation embryos. To further understand the effects of harm reduction cigarettes on early development, we investigated dynamic cellular processes such as attachment, survival, growth, and apoptosis of mESC and hESC during exposure to MS or SS smoke from both conventional and harm reduction cigarettes.
The specific purposes of this study were: (1) to establish toxicological assays using mESC and hESC as novel models for pre-implantation embryos, and (2) to use these assays to assess and compare the effects of MS and SS smoke from conventional (Marlboro Red) and harm reduction (Marlboro Lights, Advance Lights, and Quest) cigarettes.
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CHAPTER 2

METHODS FOR CULTURING MOUSE AND HUMAN EMBRYONIC STEM CELLS
Abstract

Mouse embryonic stem cells (mESCs) were derived and cultured almost 30 years ago and have been valuable tools for creating knock-out mice and for studying early mammalian development. More recently (1997), human embryonic stem cells (hESCs) have been derived from blastocysts, and numerous methods have evolved to culture hESCs in vitro in both complex and defined media. hESCs are especially important at this time as they could potentially be used to treat degenerative diseases and to access the toxicity of new drugs and environmental chemicals. For both human and mouse embryonic stem cells, fibroblast feeder layers are often used at some phase in the culturing protocol. The feeders - often mouse embryonic fibroblasts (mEFs) - provide a substrate that increases plating efficiency, helps maintain pluripotency, and facilitates survival and growth of the stem cells. Various protocols for culturing embryonic stem cells from both species are available with newer trends moving toward feeder free and serum free culture. The purpose of this chapter is to provide basic protocol information on the isolation of mouse embryonic fibroblasts and establishment of feeder layers, the culture of mESCs on both mEFs and on gelatin in serum containing medium, and the culture of hESCs in defined media on both mEFs (hESC culture medium) and on Matrigel (mTeSR). These basic protocols are intended for researchers wanting to develop stem cell research in their labs. These protocols have been tested in our laboratory and work well. They can be modified and adapted for any user's particular purpose.
1. Introduction

The first derivation of mouse embryonic stem cells (mESCs) was reported independently in 1981 by laboratories in the USA\(^1\) and in England\(^2\). Success in deriving and culturing mESCs grew from prior experience with teratocarcinoma cells, which required a feeder layer for survival and growth, and the use of fibroblast feeder layers became a standard for the derivation and growth of mESCs\(^3\). Feeder layers, which are still often used today with mESCs, have been valuable in providing conditions that support survival, proliferation, and the maintenance of pluripotency in stem cell populations. Since their initial isolation in 1981, numerous labs derived new mESC lines and used them to study mammalian development and to create knock-out mice\(^4\). Various protocols are now available for culturing mESCs and to some extent specific protocols work best with lines derived from specific strains of mice\(^3\).

In 1998, Dr. Thomson's laboratory at the University of Wisconsin reported, for the first time, that embryonic stem cells can be derived from human blastocysts and propagated \textit{in vitro}, opening the possibility of creating pluripotent cell lines with the potential to treat and cure numerous human diseases\(^5\). Initially, culturing protocols for human embryonic stem cells (hESCs) involved the use of feeder layers as substrates and media containing serum and animal proteins. However, feeder layers add complexity to stem cell cultures and have the potential to introduce animal viruses and unwanted immunogens into the stem cell populations, which would preclude the use of hESCs grown on feeders in therapeutic applications.
Subsequently, various protocols have been developed for feeder-free culturing of hESCs. The first of these replaced the feeders with Matrigel\textsuperscript{6} and replaced serum with medium conditioned by murine embryonic fibroblasts (mEFs)\textsuperscript{7}. While Matrigel is readily available and easy to use, it is not strictly defined, may vary from lot to lot, and contains animal proteins. There has thus been interest in refining the substrate, and other options, such as laminin and fibronectin, have been successful\textsuperscript{7,8}. Media conditioned by fibroblasts or media containing serum have been valuable in initial work on embryonic stem cells, but there is a trend to eliminate these undefined components, especially from hESC culture protocols. Serum is highly variable from lot to lot and in some instances may promote differentiation rather than pluripotency of embryonic stem cells. Likewise, media conditioned by fibroblasts is not defined and contributes animal proteins to the culture milieu. New defined media formulations (containing some animal proteins) that work well with embryonic stem cells, such as Knockout Serum Replacement (SR or KSR) (Invitrogen) and mTeSR (StemCell Technologies), have been developed recently\textsuperscript{9,10} and are commercially available at affordable prices. More refined xenofree media can also be purchased at a higher cost for hESC work demanding animal protein free media. Recently, three dimensional culture systems, which would be beneficial for differentiation of hESCs, have also been introduced\textsuperscript{11-13}. Culture methods for ESCs are continually evolving, and protocols can be expected to improve and become more accessible in the future.
While many different protocols have been developed for both mESCs and hESCs and while the exact choice of media and culture conditions will be determined by the needs of individual investigators and purpose of their work, we describe in this review the fundamental methods needed for isolating mouse embryonic fibroblasts (mEFs) that can be used as feeder layers for both mouse and human ESCs. We also present a standard method for culturing mESCs in a serum containing medium. It is possible to grow mESCs in serum free knock-out medium, and this would be an alternative strategy that might be preferable depending on the research goal. Finally, we present methods for growing hESCs on both mEFs and Matrigel in defined conditions using hESC medium in which serum is replaced by KSR or a new medium, mTeSR, developed by Ludwig and Thomson. We have had good success with hESC culture medium and mTeSR medium, both of which can be used with induced pluripotent stem cells (iPSCs) as well as hESCs. This set of protocols is intended for those who have not worked with ESCs before and need a starting point for accomplishing basic steps that would lead to setting up embryonic stem cell experiments in their laboratory. We present protocols as we often perform them in our laboratory. Amounts and sizes of preparations can be scaled up or down as needed. Once these methods have been mastered, numerous other techniques can be used in conjunction with ESC or more advanced methods of culturing can be added to this starting repertoire.
2. Materials

2.1. General Considerations

All methods described in this chapter need to be done using strict sterile technique. For more information on sterile technique, see the reviews by Phelan and by Cote\textsuperscript{14,15}. For more information on contamination, how to prevent it, and how to deal with it when it occurs, see the excellent technical bulletin by Ryan\textsuperscript{16}. Testing for Mycoplasma infection of cultures should be done routinely\textsuperscript{16}. We recommend that all methods be done in a clean room, which is accessed only by users of the room. Individuals entering the clean room should step onto a sticky mat, and then put on lab coats, booties, and masks, all of which are always kept in the clean room (disposable sticky mats, lab coats, latex gloves, face masks, and booties can be purchased from internet sources at considerable savings).

Floors should be cleaned at least once a week by users. Friday is a good time for this so the clean rooms are ready to use at the beginning of the next week. All items that are removed from the sterile hoods (e.g., centrifuge tubes) should be sprayed with 75\% ethanol, which is allowed to evaporate before returning them to the hood. Controlling contamination will be easier if air entering the rooms is HEPA filtered. The need for fastidious sterile technique is especially important for hESCs, which are usually cultured without antibiotics.
2.2. Culturing Mouse Embryonic Fibroblasts (mEFs)

2.2.1. Isolating mEFs

1. Ethanol spray bottle (75%). All items going into the sterile hoods should be sprayed with ethanol which will help kill microorganism upon evaporation.

2. Stereoscopic (dissecting) microscope.

3. Pipet-Aid (i.e. Drummond).

4. Sterile plastic pipettes (10 and 25 mL, individually wrapped).

5. Trypsin/ Ethylenediaminetetraacetic acid (EDTA). 40 mL of 0.25% trypsin is used for isolating mEFs, while 0.05% trypsin/EDTA solution is used for passaging cells.

6. Sterile stir bar and stir plate. Sterilize by allowing 75% ethanol to evaporate from their surfaces.

7. Autoclaved 100 mL Erlenmeyer flask (for use with 1-2 mice for isolating mEFs).

8. Autoclaved aluminum foil (small piece to cover Erlenmeyer flask).

9. Sterile deionized water (dH2O).

10. Phosphate buffered saline (PBS) without Mg2+ and Ca2+ (2600 mL dH2O, 8.3 mg NaH2PO4·H2O, 28.4 mg Na2HPO4, pH = 7.4). Autoclave and store at 4°C.

11. 0.2% gelatin (i.e. Sigma, tissue culture grade) in PBS.

12. T75 cm² tissue culture flasks. When isolating mEFs, generally 4-5 T75 cm² flasks are needed for each pregnant mouse. When passaging mEFs or mESCs, we normally use T25 cm² flasks.
13. 1 or 2 pregnant female mice. For isolating mEFs, mice should be used between day 12.5 to 13.5 of pregnancy (day 1 = first day after mating) (see Note1).


15. Sterile dissecting tools (scissors, two pairs of forceps, scalpel). Sterilize by autoclaving.

16. Sterile 60 mm diameter Petri dishes (i.e. Falcon, Fisher Scientific).

17. DNase (100 µg/mL) (i.e. Sigma, 10 mg/mL at 10,000 total units).

18. Heat inactivated and filtered fetal bovine serum (FBS): Thaw FBS in a 37°C water bath (if frozen) until serum is liquid. Gently shake, then incubate at 56°C with whole volume of serum immersed in water bath for 30 minutes (do not immerse cap). Swirl every 5-10 minutes and when done, cool in an ice bath. Do not over heat. Serum should be filtered (0.2 µm filter), aliquoted, and stored frozen at -20°C until needed.

19. Stericup filter flask (0.22 µm, 150 mL or larger depending on volume of medium being made).

20. mEF medium: 500 mL of high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM), 6 mL of 200mM L-glutamine (Invitrogen), 6 mL of penicillin/streptomycin (5000 units of penicillin + 5mg/ml streptomycin), and 55 mL of heat inactivated FBS. After combining ingredients, sterilize using a 0.22 µm filter flask and store at 4°C for up to 2 weeks. Medium can also be made in smaller batches.

21. 100 µm cell strainer (BD Falcon, 100 µm nylon).
22. Sterile 50 mL conical tubes.

23. Inverted microscope (phase contrast or Hoffman modulation).

2.2.2. Freezing and Passaging mEFs

1. Ethanol spray bottle (75%). All items going into the sterile hoods should be sprayed with ethanol which will help kill microorganism upon evaporation.

2. Cryovials (1.8 mL).

3. mEF freezing medium: 20% dimethyl sulfoxide (DMSO) and 80% mEF medium (see Note 2).

4. 0.25% trypsin/EDTA (Invitrogen).

5. mEF medium (see above).

6. Sterile 50 mL conical tubes.

7. T25cm² Nunc flasks (Fisher).

8. 0.2% gelatin (i.e. Sigma, tissue culture grade) in PBS (see Note 3).

9. Sterile 15 mL conical tubes.

10. Pipettes (P1000, P200, P20) and sterile tips appropriate for each pipette.

2.2.3. Preparing mEF Feeder Layers for Subsequent Culture of mESCs

1. mEFs isolated from these animals or mEFs purchased from commercial sources (e.g., ATCC, StemCell Technologies) which will be used to make feeder layers for mESCs and hESCs.

2. 0.2% gelatin (i.e. Sigma, tissue culture grade) in PBS.

3. Ethanol spray bottle (75%). All items going into the sterile hoods should be
sprayed with ethanol which will help kill microorganism upon evaporation.

4. mEF medium (see above).

5. Sterile 50 mL conical tubes.

6. T25cm² Nunc flasks (Fisher).

7. Sterile 15 mL conical tubes.

8. Pipettes (P1000, P200, P20) and sterile tips appropriate for each pipette.

9. Radiation source that can produce 8,000 rads

10. Leukemia inhibitory factor (LIF, Millipore), see Note 4.

11. Low LIF mESC medium: 125 mL DMEM, 22.5 mL FBS (various sources), 1.5 mL of 100mM sodium pyruvate (Invitrogen), 1.5 mL of 100x non-essential amino acids (ATCC, catalog #30-2116), 1.5 mL of 200mM L-glutamine, 750 µL penicillin/streptomycin (5000 units of penicillin + 5mg/ml streptomycin), 1 µL of 2-mercaptoethanol (>=99%, tissue culture grade, Sigma, Catalog #M7522), and 0.4 µL of LIF from the stock bottle (10⁶ units/ml).

12. Mitomycin C. Reconstitute mitomycin C in PBS (without Mg and Ca) and 5% DMSO to reach a final concentration of 1 mg/mL (e.g., 0.1 mL DMSO + 1.9 mL PBS + 2 mg mitomycin C). Mix ingredients carefully until all mitomycin C powder has dissolved (see Note 5 and 6).

13. mEF medium + mitomycin C: For each T25 cm² flask of mEFs, 4 mL of mitomycin C containing medium is used. The final concentration of mitomycin C is 10 µg/mL (e.g., for every 10 mL of mEF medium, add 100 µL of aliquoted mitomycin C solution).
14. Waste beaker (500 mL).
15. 0.05% trypsin/EDTA solution.
16. Sterile 15 mL conical tubes.

2.3. Culturing Murine Embryonic Stem Cells (mESCs)
1. Mitotically inactivated mEF feeder layers growing in T25 cm² flasks.
2. Murine embryonic stem cells (e.g., the D3 line from ATCC).
3. Low LIF mESC medium (see above).
4. Ethanol spray bottle (75%).
5. Sterile 15 mL conical tubes.
6. Pipettes (P1000, P200, P20) and sterile tips appropriate for each pipette.
7. mESC freezing medium: mESC medium plus 10% FBS, and 10% DMSO. For one T25 cm² flask, 4 mL of medium should be sufficient. (The amount can be scaled up if more than one flask is used or if a larger flask is used).
8. 0.2% gelatin (i.e. Sigma, tissue culture grade) in PBS.
9. PBS without Mg²⁺ and Ca²⁺ (see above).
10. 0.05% trypsin/EDTA solution.
11. High LIF mESC medium: as described above for low LIF mESC medium, except for LIF, make a small amount of working solution by diluting stock solution (10⁶ units/ml) 10 fold, and add 1µL of working solution for each mL of high LIF mESC medium (e.g., use 10 µL of working solution for 10 mL of mESC medium).
2.4. Culturing Human Embryonic Stem Cells (hESCs)

1. 0.05% trypsin/EDTA solution.

2. Gelatin-coated T25 cm² tissue culture flasks and 6-well plates (see Subheading 3.1., step 5 and 23).

3. hESCs (e.g., H9 line from WiCell). This could be a frozen vial or live culture.

4. Human basic fibroblast growth factor (hbFGF, i.e. Peprotech): Stock solution is made by dissolving 10 µg of hbFGF in 5 mL of PBS with 0.1% BSA (Fraction V). hbFGF is very sticky, so when using, pre-wet all pipette tips, tubes, and the filter with PBS + 0.1% BSA. Stock solution should be aliquotted (250 µl) and stored at -20°C (short term) or at -80°C (long term). Once thawed, aliquots can be stored at 4°C for a month.

5. hESC medium: 400 mL DMEM/F-12, 100 mL Knockout Serum Replacement (KSR, Invitrogen), 5 mL non-essential amino acids (NEAA, Invitrogen, catalog #11140-050), 5 mL L-glutamine-2-mercaptoethanol mix (7 µL 2-mercaptoethanol plus 5 mL of 200mM L-glutamine), and 1 mL of hbFGF (10µg/5ml of PBS with 01% BSA) to a final concentration of 4 ng/mL. Filter through a 0.22 µm filter and store at 4°C (it is stable for 2 weeks). It can be made in smaller batches.

6. Ethanol spray bottle (75%).

7. Sterile 15 mL conical tubes.

8. Matrigel™.

9. DMEM/F-12.
10. mTeSR culture medium kit (includes basal medium plus supplements) (StemCell Technologies). Medium is made by combining 400 mL of mTeSR basal medium with 100 mL of mTeSR supplement. Complete medium can be made in smaller aliquots. Complete medium is stable at 4°C for 2 weeks.

11. PBS without Mg²⁺ and Ca²⁺ (see above).

12. Accutase (eBioscience) or collagenase IV or trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA). Collagenase IV is made by warming DMEM/F12 in a 37°C water bath, then dissolving 0.01 mg of collagenase per mL of DMEM/F12. The solution is passed through a 0.22 µm filter before use.

13. Glass beads (3 mm diameter, i.e. Fisher Scientific). To prepare beads, place a bottle of glass beads in a beaker (beaker should be ⅓ to ½ full). Rinse the beads three times with dH₂O, then cover the beads with 10 N HCl and soak overnight at room temperature. Add an equal volume of 10 N NaOH (carefully) to neutralize HCl, then run dH₂O over beads overnight. The next day remove all water from the beaker and wash four times with dH₂O. Dry off glass beads with an autoclaved paper towel or in drying oven and put bead back in beaker and cover with aluminum foil. Autoclave the beaker/beads and glass test tubes with plastic snap on caps. Allow beads to dry overnight, then aliquot the beads into the glass test tubes. Autoclave the test tubes with the beads and allow them to dry overnight, after which they will be ready for use in passaging hESCs.
14. Sterilized inverted light microscope. Sterilize with 75% ethanol and UV light in hood.

15. Sterile scalpel.

16. Sterile 10 mL Pasteur pipettes.
3. Methods

3.1. Culturing mEFs

3.1.1. Procedure for Isolating mEFs

1. Place 75% ethanol sterilized dissection microscope in the sterile hood.

2. Wipe down all bench tops that will be used with 75% ethanol.

3. Place 40 mL of 0.25% trypsin and a sterile stir bar in the Erlenmeyer flask, cap the flask with autoclaved aluminum foil, and pre-heat trypsin solution in a 37°C water bath.

4. Pre-heat 40 mL of mEF medium in a 50 ml conical tube in the 37°C water bath.

5. Coat T75 cm² culture flasks with 6 mL of 0.2% gelatin (see Note 7) and incubate at 37°C for a minimum of 15 minutes or until needed (see Note 3).

6. Sacrifice female mice (one at a time) using CO₂ gas.

7. Place sacrificed female mouse on her back on autoclaved paper towels.

8. Spray the mouse with 75% ethanol.

9. Open the peritoneal cavity by making a Y-incision (Fig. 2.1).

10. Dissect out embryos from the uterus and remove all tissue surrounding each embryo using sterile forceps.

11. Carefully transfer dissected embryos into 60 mm Petri dishes containing sterile PBS.

Fig. 2.1 Diagram showing where to make incisions on the ventral surface of a mouse.
12. Rinse embryos in clean PBS twice and place them in new dish with fresh PBS for dissection.

13. Remove the head and internal organs from the embryos carefully with sterile forceps and scissors.

14. Place all dissected embryos in a fresh 60 mm dish of PBS and cut tissue into approximately 1 mm pieces with the scalpel.

15. Transfer all tissue pieces into the sterile Erlenmeyer flask containing 40 mL of pre-heated (37°C) 0.25% trypsin/EDTA solution and sterile stir bar.

16. Stir cell solution for 40 minutes at room temperature on a stirring plate.

17. Observe solution periodically. Add 200 µL of 100 µg/mL DNase, if solution appears viscous and clumpy (add additional DNase in 200 µL increments if necessary).

18. After 40 minutes, add 40 mL of pre-warmed (37°C) mEF medium and swirl solution gently.

19. Strain the solution through a 100 µm cell strainer into a 50 mL conical tube in the sterile hood. Repeat this procedure twice. Use a new strainer each time.

20. Centrifuge the cell suspension in the 50 mL conical tube at 270g for 4 minutes.

21. Decant supernatant into a fresh sterile 50 mL conical tube (save the supernatant in case there are not enough cells for plating).

22. Re-suspend the pellet using fresh mEF medium (1 mL for each T75 cm² flask, e.g., if using 4 flasks, break the pellet with 4 mL of medium).

23. Aspirate excess gelatin out of T75 cm² flasks and replace it with 8 mL/flask of
mEF medium.

24. Add 1 mL of cell suspension to each T75 cm² flask, and rock the flask back and forth gently to distribute cells evenly across the bottom of the flask.

25. Observe cells with the inverted microscope to make sure they look normal, for example, round with smooth surfaces and not apoptotic (see Note8).

26. Incubate cells in the 37°C incubator.

27. Change medium after 24 hours of plating, and thereafter on alternate days.

28. Allow cells to reach 90-95% confluency (which should take 3-4 days) before passaging or freezing (Fig. 2.2B). (See Note 9 and 10).

3.1.2. Freezing and Storing Passage 1 mEFs

1. Label cryovials with cell line, passage number, date, and initials (see Note 11).

2. Aspirate mEF medium and wash the cells twice with 5 mL of room temperature PBS.

3. Aspirate PBS and add 5 mL of 0.05% trypsin/EDTA to each T75 cm² flask and incubate at 37°C for 1 minute.
4. Remove flasks from incubator and gently tap the sides of each flask. Do not leave cells in trypsin for more than 5 minutes (see Note 12).

5. Add 10 mL of mEF medium to each flask and mix gently. Amount of mEF medium added should be double the amount of trypsin solution that is used.

6. Aspirate the cell suspension from each flask and transfer it to one sterile 50 mL conical tube.

7. Centrifuge the cells at 270g for 4 minutes, then decant off the supernatant.

8. Break the pellet with 5 mL of freezing medium, then add the remaining 5 mL of the freezing medium and mix by gently inverting the tube (e.g., for five T75 cm² flasks, the total volume of freezing medium will be 50 mL).

9. Add 1 mL of the cell suspension in freezing medium to each sterile cryovial.

10. Transfer the cryovials to a -80°C freezer and leave overnight.

11. Remove cryovials from freezer, place on dry ice, and transfer to liquid nitrogen for long term storage (see Note 13).

3.1.3. Preparing and Freezing Passage 2 mEFs

1. It is convenient to grow and freeze a number of passage 2 vials of mEFs for eventual use as feeder layers (see Notes 14 and 15).

2. Coat 3-4 T25 cm² flasks with 0.2% gelatin and incubate for at least 15 minutes.

3. Get a passage 1 vial of mEFs from the liquid nitrogen storage and bring the vial to a 37°C water bath on dry ice (see Note 16).
4. Thaw the cryovial in the water bath for no longer than 1.5 minutes or until a small ice crystal is left (see Note 17).

5. Spray the cryovial with 75% alcohol and transfer it to the sterile hood. Allow alcohol to evaporate off before opening the vial.

6. Place 5 mL of fresh mEF medium into a 15 mL conical tube.

7. Transfer thawed cells into the conical tube using the P1000 pipette.

8. Rinse cryovial with 1 mL of mEF medium and transfer to the conical vial.

9. Cap the tube and centrifuge for 4 minutes at 270g. Spray the tube with 75% ethanol before transferring it to the sterile hood.

10. Decant supernatant and break the pellet with 3-4 mL of fresh mEF medium.
    Use 1 mL of mEF medium per T25 cm² flask.

11. Aspirate gelatin from the T25 cm² flasks.

12. Add 3 mL of fresh mEF medium into each T25 cm² flask.

13. Plate 1 mL of mEFs into each T25 cm² flask, rock flask gently to distribute cells, and incubate at 37°C. Observe flasks after 20 minutes to verify that cells are attaching.

14. Change the medium 24 hours after plating, then change the medium on alternate days.

15. When 90 - 95% confluency is reached, a flask may be used to prepare the feeder layer (Fig. 1.2B). Cells in the remaining flasks can be frozen as was done for passage 1 cells (Subheading 3.1.2.).
3.1.4. Preparing mEF Feeder Layers for Subsequent Culture of mESCs

1. Coat T25 cm\(^2\) flasks with 0.2% gelatin and incubate for at least 15 minutes (use two flasks for each vial of mEFs).

2. Get stock vial of passage 2 mEFs from liquid nitrogen storage or use a T25 cm\(^2\) flask containing a live culture of passage 2 mEFs (see Subheading 3.1.3. and Notes 14 - 16).

3. If frozen, thaw cells in the 37°C water bath for 1.5 minutes or until only a small crystal of ice remains. Keep cap above water during thawing.

4. Before transferring to the sterile hood, spray vial with 75% ethanol.

5. In a sterile hood, transfer thawed cells (1 mL) to a 15 mL conical tube containing 5 mL of mEF medium using a P1000 pipette. Rinse cyrovial with 1 mL of mEF medium and add this to the conical tube.

6. Centrifuge at 270g for 4 minutes to create a loose pellet of cells.

7. Decant supernatant from the conical tube and gently break the pellet with 1-2 mL of mEF medium per pellet.

8. Aspirate excess gelatin from the T25 cm\(^2\) flasks.

9. Pipette 0.5 to 1.0 mL of mEF medium containing the cell suspension into each of two T25 cm\(^2\) flasks containing 3 mL of fresh medium and transfer flasks to the incubator.

10. After about 15-20 minutes, check to be sure that mEFs are attaching to the bottom of the flasks using an inverted microscope. Then observe the flasks daily for cell growth, normal appearance, and absence of contamination.
11. Cells will double about every 24 hours. For use with mESCs, let mEFs grow until they are about 90-95% confluent.

12. mEF medium should be changed every other day.

3.1.5. Mitotic Inactivation of mEFs Using Irradiation

1. To prevent mEFs from dividing when stem cells are plated on them, they must be inactivated with either irradiation (for example cesium exposure) or with mitomycin C (see Note 18).

2. To irradiate mEFs, place flasks in a cesium source irradiator and irradiate at 8,000 rads for approximately 2 hours. It may be necessary to empirically determine the length of exposure and dosage for your particular instrument.

3. Remove flasks promptly and return them to the cell culture room.

4. Upon return to the lab, immediately replace mEF medium with 4 mL of room temperature low LIF mESC medium and incubate at 37°C for 30-60 minutes, after which mEFs can be used as feeder layers (see Notes 19 and 20).

3.1.6. Mitotic Inactivation of mEFs Using Mitomycin C

1. If an irradiator is not available, mEFs can be inactivated using mitomycin C.

2. Label a 50 mL conical vial to dispose of any waste containing mitomycin C.

3. Aspirate mEF medium out of T25 cm² flasks containing mEFs, add 4 mL of mEF medium + mitomycin C into each flask, and incubate at 37°C for 2 to 2.5 hours.

4. For each T25 cm² flask of mitomycin treated mEFs, coat one T25 cm² flask with gelatin and incubate for at least 15 minutes.
5. After treatment, aspirate the mitomycin C containing mEF medium out of the T25 cm² flask (see Note 5).

6. Wash mitomycin C treated mEFs with 5 mL of PBS for three times and discard the wash into the waste bottle containing mitomycin C medium.

7. Add 2 mL of 0.05% trypsin/EDTA to each T25 cm² flask and incubate at 37°C for 1 minute.

8. Remove the flasks from the incubator and gently tap the sides of the flasks to loosen the cells. Check the flasks using the inverted microscope periodically to make sure mEFs are detaching from the bottom of the flask. Do not leave mEFs in trypsin for more than 5 minutes (see Note 12).

9. Add 4 mL of mEF medium to each flask to inactivate the trypsin.

10. Transfer the 6 mL of cell solution from each flask into a single sterile 15 mL conical tube and cap the tube.

11. Centrifuge the cell solution at 270g for 4 minutes. Spray conical tube with 75% ethanol before returning it to the sterile hood.

12. Decant the supernatant and gently break pellet with 1-2 mL of fresh mEF medium by pipetting repeatedly with a P1000 pipette. Use 1 mL of mEF medium per flask being plated.

13. Aspirate excess gelatin in T25 cm² flasks and add 3 mL of mEF medium to each flask.

14. Plate mitomycin C treated mEFs on two fresh 0.2% gelatin coated T25 cm² flasks. Observe plated cells after 20 minutes (see Note 21).
15. mEFs freshly treated with mitomycin C can be used as feeders after overnight incubation (see Note 22).

16. mEF medium should be changed 24 hours after mitomycin C treatment and every other day thereafter.

3.1.7. Schedule for Preparing mEFs

1. It is a good idea to set up a schedule for harvesting mEFs so that you will have them ready when needed for your stem cells. An example of a possible schedule is given in Table 2.1.

<table>
<thead>
<tr>
<th>Day of week</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monday</td>
<td>Plate passage 2 mEFs on gelatin coated T25 cm² flasks.</td>
</tr>
<tr>
<td>Tuesday</td>
<td>Change medium for newly plated mEFs.</td>
</tr>
<tr>
<td>Wednesday</td>
<td>Observe mEFs using a inverted light microscope for confluency and contamination.</td>
</tr>
<tr>
<td>Thursday</td>
<td>Mitomycin C treat or irradiate mEFs. Irradiated mEFs can be used on Thursday for plating mESCs.</td>
</tr>
<tr>
<td>Friday</td>
<td>Mitomycin C inactivated mEFs can be used for plating ESCs; change medium if not used.</td>
</tr>
</tbody>
</table>

Table 2.1. Schedule for mEF Preparation.

2. All mitotically inactivated mEFs should be fed on alternative days, and they are good for 2 weeks.

3.2. Culturing mESCs

3.2.1. Thawing and Expanding mESCs on mEFs

1. Prepare mEF feeder layer as described in Subheadings 3.1.4. to 3.1.6.

2. Aspirate mEF medium off of mEFs and add fresh low LIF mESC medium (4 mL to a T25 cm² flask) then incubate for at least 30-60 minutes (see Note 23).
3. Get a vial of frozen mESCs from liquid nitrogen storage and transport it on dry ice to the cell culture room.

4. Immediately put the frozen vial in the 37°C water bath without submerging the cap and thaw for no more than 90 seconds or until a small ice crystal is left.

5. Remove vial and spray down with 75% ethanol before placing it in the sterile hood.

6. Put 5 mL of fresh low LIF mESC medium in a 15 mL conical tube.

7. Transfer the mESCs into the conical tube using the P1000 pipette, then wash the vial with 1 mL of low LIF mESC medium and add wash to the tube. Cap the tube before removing it from the hood.

8. Spin down cells in centrifuge at 270g for 4 minutes, then spray the conical tube with 75% ethanol before returning it to the hood.

9. Decant the supernatant into the waste beaker, and add 1 mL of low LIF mESC medium to the tube. Pipette gently with the P1000 pipette to break the pellet. Be sure the cells are fully dispersed, but do not pipette too hard or the cells may be damaged.

10. Set the cells aside and aspirate the low LIF mESC medium out of the T25 cm² flask and replace it with fresh low LIF mESC medium (4 mL).

11. Add 1 mL of mESC suspension to the T25 cm² flask using a P1000, then rock the solution in the flask back and forth gently to evenly distribute the cells. Observe using the inverted microscope and be sure the cells appear normal and are not apoptotic.
12. Incubate at 37°C and check the next day for evidence of mESC attachment.

13. Observe cultures every day to be sure there is no evidence of contamination or differentiation (see Note 24).

14. Change the mESC medium every day (replace 5 mL of old medium with 5 mL of fresh low LIF mESC medium).

15. For vials that are freshly thawed, it will generally take 3-4 days for mESCs to become 50-60% confluent (adjacent colonies should not be touching each other).

16. Figs. 2.3A and B show examples of pluripotent mESCs growing in colonies. These colonies are round, three dimensional, and have defined edges. Colonies in Fig. 1.3B have been labeled to show alkaline phosphate activity, a marker for pluripotency. Figs. 2.3C and D, in contrast, show mESC colonies in which...
differentiation has begun. Colonies are flatter and cells (arrow) have begun to migrate out of a colony.

### 3.2.2. Passaging and Freezing mESCs using mEFs

1. Aspirate mEF medium from a new flask of mitotically inactivated mEFs, add 4 mL of low LIF mESC medium to the flask, and incubate at 37°C for 30 to 60 minutes.

2. Coat a 60 mm tissue culture dish with 0.2% gelatin for at least 15 minutes and incubate at 37°C until ready for use.

3. Aspirate the low LIF mESC medium out of the T25 cm² flask containing mESCs.

4. Add 5 mL of PBS to wash any serum out of the flask.

5. Aspirate out PBS and add 2 mL of 0.05% trypsin/EDTA to the flask with mESCs, then incubate for 1 minute at 37°C (see Note 12).

6. Remove the flask from the incubator and gently tap its sides to loosen the cells. Check using the inverted microscope to be sure cells have detached.

7. Add 4 mL of low LIF mESC medium to the flask with mESCs and rock back and forth (flask contains a total of 6 mL).

8. Aspirate out 1 mL of solution containing mESCs using a P1000 pipette and transfer to a 15 mL conical tube and cap tube.

9. Aspirate out the remaining 5 mL of solution containing mESCs and transfer to a second 15 mL conical tube and cap tube.

10. Centrifuge the two conical tubes for 4 minutes at 270g. Spray down with 75%
ethanol before placing tubes back in the hood.

11. Decant medium from conical tube containing 5 mL, and gently break the pellet with 4 mL of freezing medium.

12. Add 1 mL of cell suspension in freezing medium per cyrovial (4 vials total for one T25 cm² flask).

13. Transfer to a -80°C freezer for 24 hours, then transfer to liquid nitrogen (use dry ice to carry vials from freezer to liquid nitrogen tank).

14. Decant the medium from conical tube containing 1 mL of solution.

15. Break the pellet gently with 1 mL of low LIF mESC medium and set aside in the hood.

16. Aspirate out excess gelatin from a 60 mm culture dish and add 3 mL of fresh low LIF mESC medium.

17. Add 1 mL of the mESC suspension to the 60 mm culture dish using a P1000 pipette and mix solution carefully with the pipette.

18. Place the 60 mm culture dish in the incubator and wait 20-30 minutes to allow mEFs to attach (see Note 25).

19. After 20-30 minutes, aspirate the cell suspension containing mESCs from the 60 mm dish and transfer to a 15 mL conical tube and cap tube (see Note 26).

20. Centrifuge the cell suspension for 4 minutes at 270g.

21. While centrifuging, change the medium in the T25 cm² flask containing mEFs by replacing 4 mL of mESC medium with an equal amount of low LIF mESC medium. Spray the conical tube before returning it to the hood.
22. Decant the medium from the conical tube, and break the pellet using 1mL of low LIF mESC medium.

23. Transfer all of the cell suspension into a T25 cm² flask containing mEFs in fresh low LIF medium and rock the flask back and forth to distribute the cells.

24. Incubate the flasks at 37°C.

25. Routinely check and change medium everyday as described in Subheading 3.2.1.

3.2.3. Plating mESCs on Gelatin

1. Add 3 mL of 0.2% gelatin to T25 cm² flasks and incubate at 37°C for at least 15 minutes (1 T25 cm² flask of mESCs can be passaged to 3 new flasks).

2. Decant medium in the T25 cm² flask containing mESCs, and wash flask using 5 mL of PBS.

3. Aspirate PBS and add 2 mL of 0.05% trypsin/EDTA to the T25 cm² flask and incubate for 1 minute at 37°C.

4. Remove the flask from the incubator and the tap side of the flask gently. Check cells with the inverted light microscope to see if all mESCs have detached.

5. Once all mESCs have detached (which should be no longer than 5 minutes), add 4 mL of high LIF mESC medium to neutralize the trypsin. (see Notes 12 and 27).

6. Aspirate cell suspension and transfer to a sterile 15 mL conical tube and cap tube.
7. Centrifuge mESCs at 270g for 4 minutes.
8. While centrifuging, remove gelatin coated flasks from incubator and decant excess gelatin in sterile hood.
9. Add 3 mL of high LIF mESC medium to the flask and set in hood (see Note 27).
10. Decant supernatant and break pellet gently with 3 mL of high LIF mESC medium.
11. Add 1 mL of cell suspension to each flask and rock flasks back and forth for even distribution. Observe cells using an inverted light microscope for normal cell morphology (round, no apoptosis).
12. Incubate mESCs at 37°C and change medium every day.
13. mESCs should be ready for passaging once flask reaches 70-75% confluency (Fig. 2.2A).
14. Fig. 2.3E shows pluripotent mESCs plated on gelatin. The colony is flat and has the characteristic cobblestone appearance of mESCs on gelatin. Fig. 2.3F shows mESCs that are starting to differentiate on gelatin. Cells have begun migrating out of the colony.
15. Once a frozen vial of mESCs has been expanded and passaged to produce sufficient frozen stock, experiments can be set up with the mESCs.
3.3. Culturing Human Embryonic Stem Cells (hESCs)

3.3.1. Prepping mEFs for hESC Culture using Mitomycin C

1. Plate a vial of frozen mEFs (passage 2) in a gelatin coated T25 cm$^2$ flask (see Subheading 3.1.1. and Note 14).
2. Allow the mEFs to grow to 95-100% confluency (Fig. 2.2B).
3. Mitomycin C treat the T25 cm$^2$ flask of mEFs (see Subheading 3.1.6.).
4. After treatment, wash the flask twice with PBS, then remove the mEFs using trypsin/EDTA (see Subheading 3.1.6.).
5. Centrifuge the cell suspension, remove the supernatant, and break pellet with fresh mEF medium as described in Subheading 3.1.6.
6. One T25 cm$^2$ flask of mitomycin C treated mEFs can be replated into 4-5 wells of a 6-well plate (this will give about 70% confluency in each well) (Fig. 2.2A).
7. Alternatively, the mitomycin C treated mEFs can be placed back into a T25 cm$^2$ flask if desired.
8. Put the plate or flask in the incubator for 24 hours (check after 20 minutes to be sure cells begin to attach).
9. After 24 hours, the mEFs are ready to be used for plating hESCs.

3.3.2 Prepping mEFs for hESC Culture using Irradiation

1. Plate two vials of frozen passage 2 mEFs in a gelatin coated 6-well plate. Two vials contain enough cells for 6 wells.
2. Allow mEFs to grow to 70-75% confluency in the incubator (Fig. 2.2A)
3. Irradiate as described in **Subheading 3.1.5.**

### 3.3.3 Thawing and Plating hESCs on mEFs

1. For the mitomycin C treated or the irradiated mEFs, replace mEF medium with hESC medium and incubate for 30-60 minutes at 37°C. Use 1 mL for each well of a 6-well plate or 3 mL into a T25 cm² flask.

2. Obtain a frozen vial of hESCs from liquid nitrogen storage (see **Notes 16 and 28**). One frozen vial of hESCs is usually plated in one well of a 6-well plate or in one T25 cm² flask.

3. Thaw the vial in 37°C water bath for no more than 1.5 minutes or until a small crystal is left (do not submerge the cap). Spray vial with 75% ethanol before transferring to sterile hood.

4. Put 5 mL of fresh hESC medium into a 15 mL conical tube.

5. Add hESCs to the conical tube drop wise by running drops down side of tube.

6. Centrifuge the tube at 200g for 3 minutes at room temperature. Spray the tube with 75% ethanol before returning it to the sterile hood.

7. Decant the supernatant and gently break the pellet using 1 mL of hESC medium.

8. Replace the hESC medium in the 6-well plate (1 mL) or flask (3 mL) with fresh hESC medium.

9. Add the 1 mL of hESC suspension drop wise into one well of a 6-well plate or one T25 cm² flask.
10. Routinely check and change medium every day.

11. Fig. 2.4A shows hESCs growing on mEFs. The hESCs actually grow in between the mEFs and form a colony. The colony is flat, has a cobblestone appearance, and well defined edges.

**3.3.4. Preparing Matrigel Plates for hESCs**

1. Thaw a 5 mL bottle of stock Matrigel in the refrigerator overnight. Do not thaw at room temperature because it may clump and not be usable.

2. Add 5 mL of DMEM/F12 (at 4°C) to the Matrigel bottle and mix thoroughly by pipetting up and down gently (avoid formation of bubbles at the surface of the solution).

3. Aliquot 500 µL of Matrigel solution to twenty 15 mL conical tubes. Aliquots that will not be used should be stored at -20°C. When using a frozen aliquot, it should be thawed for 1-2 hours in the refrigerator (4°C) before use.

4. When making Matrigel plates, add 7 mL of DMEM/F12 (at 4°C) to a 500 µL aliquot of Matrigel prepared as described above and pipette gently without making bubbles.
5. Add 1 mL of Matrigel working solution to each well in a 6-well plate, and rock to evenly distribute the solution.

6. Leave the plate at room temperature for 1-2 hours or leave it in the refrigerator overnight (see Note 29).

3.3.5 Thawing and Plating hESCs on Matrigel

1. One frozen vial of hESCs is usually plated in one well of a 6-well plate or in one T25 cm² flask.

2. Thaw a vial of hESCs at 37°C for 1.5 minutes in a water bath or until a small ice crystal is left.

3. Add 5 mL of mTeSR to a 15 mL conical tube.

4. Transfer the cells in the thawed vial to the conical tube by adding cells drop wise to the side of the tube, and cap the tube.

5. Centrifuge for 3 minutes at 200g and spray the tube with 75% ethanol before placing it the sterile hood.

6. Decant the supernatant from the tube and gently break the pellet using 1 mL of mTeSR medium. Do not pipette too many times; cells should remain in colonies.

7. Aspirate out excess Matrigel from the 6-well plate and add 1 mL of fresh mTeSR to each Matrigel-coated well.

8. Add 1 mL of cell suspension to the Matrigel-coated well drop wise, keeping
drops near the surface of the well.

9. Incubate the plate at 37°C overnight.

10. Change the mTeSR medium every day.

11. Passage when hESCs are 70-75% confluent.

12. Fig. 1.4B shows a hESC colony plated on Matrigel. The colony is flat with a cobblestone appearance. The cells are tightly joined to each other in a colony.

3.3.6. Passaging hESC on Either mEFs or Matrigel-Coated Dishes with Glass Beads

1. If plating hESC on mEFs, remove mEF medium and replace with 1 mL of hESC medium, and incubate at 37°C for 30 to 60 minutes before using. If plating on Matrigel, prepare Matrigel coated dishes 1-2 hours before they are needed.

2. Wash hESCs with 1 mL of PBS per well then aspirate out PBS.

3. Add 1 mL of Accutase or collagenase IV to each well and incubate at 37°C for 1 minute (see Note 30).

4. Remove plate from incubator and observe with an inverted microscope. Edges of the colonies will start to curl.

5. Add 10-12 sterile glass beads to each well (see Notes 28 and 29).

6. Rock plate back and forth gently. Observe the plate from the bottom. hESC colonies should become detached and float in solution.

7. Do not leave hESC in Accutase or collagenase for more than 3 minutes.

8. If plating on mEFs, add 2 mL of hESC medium to the well. If plating on Matrigel,
add 2 mL of mTeSR medium to the well.

9. Aspirate the cell suspension and transfer to a 15 mL conical tube and cap the tube.

10. Centrifuge the cell suspension at 200g for 3 minutes. Spray the conical tube with 75% ethanol before placing in hood.

11. Decant supernatant and gently break the pellet using either hESC medium (for mEF plating) or mTeSR medium (for Matrigel plating). If 2 wells of hESCs will be passaged into 6 new wells, then break pellet with 3 mL of medium, or 500 μL per well.

12. Do not pipette too many times to prevent colonies from breaking up (hESCs survive better in colonies and not as single cells).

13. If plating onto mEFs, aspirate hESC medium from the wells and add 1 mL of fresh medium. If plating on Matrigel, aspirate out Matrigel from the wells and add 1 mL of mTeSR medium.

14. Replate hESCs by adding 500 μL of cell suspension drop wise to each well. Add drops right above the surface of the solution. Rock the plate in all four directions gently.

15. Clumps of hESCs should be observed using the inverted microscope.

16. Incubate the cells at 37°C overnight and change the medium (hESC medium or mTeSR) every day.

17. Passage again when colonies reach 70-75% confluency.

18. Once a frozen vial of hESCs has been expanded and passaged to produce
sufficient frozen stock, experiments can be set up with the hESCs.

### 3.3.7. ‘Cut and Paste’ Passaging

1. Instead of using the beads, the “cut and paste” method of passaging may be used. For this alternative place a sterilized inverted light microscope in sterile culture hood.

2. Place a 6-well plate of hESCs on the stage of the microscope and open the lid.

3. Observe hESC colonies and chose those that look pluripotent and healthy (Fig. 2.4A, B).

4. Use a scalpel to gently cut the colonies into quadrants (Fig. 2.5A).

5. Gently scrape off the desired portions of the colonies.

6. Once all the pieces have been mechanically removed from the plate, transfer the solution in each well into a 15 mL conical tube.

7. Centrifuge the cell suspension and break pellet with appropriate media (mTeSR if plating on Matrigel and hESC medium if plating on mEFs).

8. Replate hESC colonies as described in Subheading 3.3.6, steps 8 to 18.
3.3.8. Passaging hESCs Mechanically

1. Instead of using the beads, the mechanical method can also be used to passage cells. For this alternative place the hESC plate in the sterile hood.

2. Add 1 mL of Accutase or collagenase IV to each hESC well and incubate for 1 minute at 37°C.

3. Return the plate to the sterile hood.

4. Unwrap a 10 mL Pasteur pipette in sterile hood. Do not allow the tip to touch any surfaces.

5. Scrape the colonies off from the bottom of the wells in the directions shown in Figure 2.5B. You should see colonies floating in medium.

6. Add 2 mL of hESC medium or mTeSR medium into each well.

7. Aspirate all cell suspensions into one 15 mL conical tube and cap tube.

8. Centrifuge the cell suspension at 200g for 3 minutes.

9. Break pellet and replate hESCs on Matrigel or mEFs as described in Subheading 3.3.6, steps 8 to 18.
4. Notes

1. Although mEFs from various strains of mice have been used successfully in stem cell culture, we have found that mEFs from NIH Swiss mice work very well with both mESCs and hESCs.

2. Make 10 mL of freezing medium per T75 cm$^2$ flask of cells that will be frozen (e.g., for five flasks, make 50 mL of freezing medium).

3. When plating mEFs or mESCs, T25 cm$^2$ and T75 cm$^2$ flasks can be used with or without gelatin coating. We have found that mEFs and mESCs stick better and are healthier with a gelatin coating, and therefore we routinely coat flasks for mEF isolation or passaging.

4. LIF is expensive. It can be made in small batches so as not to waste it. The stock bottle is stored at 4°C. Check expiration date of each bottle.

5. Mitomycin C is a potent chemical that inhibits cell division. It should be handled carefully under a hood while wearing gloves. Be sure to discard mitomycin C waste in the appropriate toxic waste bin.

6. Aliquots of mitomycin C stock solution can be stored at -20°C for 6 months. The unconstituted powder is stable at -20°C for one year.

7. Cells from about 2-3 embryos can be plated on one T75 cm$^2$ flask.

8. Observe cells with the inverted microscope at 20 minutes after plating. If mEFs are healthy, they will have begun attaching at this time.

9. It normally takes the mEFs about 3-4 days to become 90% confluent. If it takes
longer than this, the cells may be unhealthy.

10. We refer to these cells as passage 1 mEFs (some labs call this stage passage 0).

11. One 90-95% confluent T75 cm\(^2\) flask of passage 1 mEFs will usually yield 10-12 frozen vials.

12. It is important not to over trypsinize the cells. To determine if cells have completely dislodged, you can examine flask carefully by eye or look at it with the inverted microscope. Do not leave mESCs in trypsin for more than 4-5 minutes. Staying in trypsin too long will reduce plating efficiency.

13. Although it is customary to store mEFs in liquid nitrogen, we have also stored mEFs at -80°C for up to 6 months with success.

14. Passages 3 or 4 are the best for use as feeder layers. We have found that younger or older passages do not work as well.

15. Our frozen vials of mEFs normally contain 3-5 × 10\(^6\) cells/vial.

16. It is important to keep cells frozen until you thaw them in the water bath. If necessary, they can be transferred from the liquid nitrogen storage site to the water bath on dry ice.

17. Do not submerge the cap.

18. The use of a cesium source machine may require special training at your institution. Try to receive training and gain access to the cesium source before beginning your work.

19. Irradiation can also be done when cells are in 15 mL conical tubes before plating on flasks as feeders.
20. Once the mEFs have been mitotically inactivated, they are only good for 10 days.

21. mEFs should begin to attach by 15 minutes after plating.

22. Irradiation of cells in flasks has the advantage that cells are ready to use after the relatively short irradiation period. Mitomycin C treated cells have the disadvantage of needing to be removed from their flasks, and replated which requires an extra night of culture before they can be used. We have used both methods with success. If mitomycin C treated mEFs have less than 70% confluency when replated, the mitomycin C treatment may have damaged some of the mEFs.

23. FBS is highly variable from batch to batch. Even embryonic stem cell qualified batches may not support pluripotency well in ESC populations. It is important to screen batches of FBS to ascertain which are suitable for your work. Often companies will hold specific lots of FBS in reserve for you if you commit to purchase them in the future.

24. Be sure to also observe the morphology of the mEFs. If mEFs start to die or detach, mESCs will not be properly supported.

25. Check dish after 15 minutes to see if most mEFs have already attached; if so the mEFs were healthy.

26. Do this step one time if passaging mESCs or two times if preparing mESCs for an experiment.
27. High LIF medium should be used when plating mESC on gelatin to keep them from differentiating.

28. A number of lines of hESCs are available for culture. Some lines require their own particular protocols. The protocol described here works well with the WiCell H9 line.

29. Matrigel plates are good for 2 weeks if refrigerated, although fresh plates are preferable.

30. Accutase will give mainly single cells, while collagenase IV will give mainly colonies.
5. References


CHAPTER 3

COMPARISON OF TOXICITY OF SMOKE FROM TRADITIONAL AND HARM REDUCTION CIGARETTES USING EMBRYONIC STEM CELLS AS A NOVEL MODEL FOR PRE-IMPLANTATION DEVELOPMENT
Abstract

BACKGROUND: Embryonic stem cells, which originate from the inner cell mass of blastocysts, are valuable models for testing the effects of toxicants on pre-implantation development. In this study, mouse embryonic stem cells (mESC) were used to compare the toxicity of mainstream (MS) and sidestream (SS) cigarette smoke on cell attachment, survival, and proliferation. In addition, smoke from a traditional commercial cigarette was compared to smoke from three harm reduction brands. METHODS: MS and SS smoke solutions were made using an analytical smoking machine and tested at three doses using D3 mESC plated on 0.2% gelatin. At 6 and 24 hours, images were taken and the number of attached cells was evaluated. RESULTS: Both MS and SS smoke from traditional and harm reduction cigarettes inhibited cell attachment, survival, and proliferation dose dependently. For all brands, SS smoke was more potent than MS smoke. However, removal of the cigarette filter increased the toxicity of MS smoke to that of SS smoke. Both MS and SS smoke from harm reduction cigarettes were as inhibitory or more inhibitory than their counterparts from the traditional brand. When pre-implantation mouse embryos were cultured for 1 hour in MS or SS smoke solutions from a harm reduction brand, blastomeres became apoptotic, in agreement with the data obtained using mESC. CONCLUSION: mESC provide a valuable model for toxicological studies on the pre-implantation stage of development and were used to show that MS and SS smoke from traditional and harm reduction cigarettes are detrimental embryonic cells prior to implantation.
1. Introduction

Embryos and fetuses are more sensitive to environmental toxicants than adults, and there is a recognized need for new assays to study the effects of environmental toxicants on prenatal stages of development (Grandjean, et al., 2007). Since “time” as much as “dose” determines chemical susceptibility in utero, it is important to develop assays that can monitor the effects of environmental chemicals at different times during prenatal development, including the pre-implantation stage. Because embryonic stem cells are derived from the inner cell mass of blastocysts (Martin, 1981, Evans and Kaufman, 1981), they represent a powerful in vitro model for studying the earliest stages of mammalian development. In this study, we have used embryonic stem cells as a toxicological model to access the effects of cigarette smoke on pre-implantation development.

Cigarette smoke was chosen as the toxicant for evaluation as it is known from numerous epidemiological studies that mainstream (MS) cigarette smoke, which is actively inhaled by smokers, can lead to a variety of adverse reproductive outcomes that include spontaneous abortion, placenta abruptio, perinatal mortality, congenital malformations, ectopic pregnancy, length of time to conceive, and decrease in birth weight and fertility rate (Andres and Day, 2000, Berthiller and Sasco, 2005, Higgins, 2002, Shiverick and Salafia, 1999, Rogers, 2008). More recently, postnatal defects in cognition, intellectual development, and behavior, as well as adverse respiratory effects, have been correlated with MS cigarette smoke exposure during pregnancy (DiFranza, et al., 2004, Perera, et al., 2006, Lannerdo, et
al., 2006, Jaakkola and Gissler, 2004). Although not as thoroughly studied as MS smoke, epidemiological evidence also indicates that SS smoke (the smoke that burns off the end of a cigarette) can adversely affect reproduction, e.g. by reducing birth weight (Hruba and Kachlik, 2000, Goel, et al., 2004, Hegaard, et al., 2006, Windham, et al., 1999) and increasing fetal mortality and preterm delivery (Kharrazi, et al., 2004).

A number of in vitro tests have been developed to study how cigarette smoke interacts with the reproductive organs and affects prenatal development (Talbot, 2008). These in vitro assays, which allow rapid testing of cigarette smoke and its components on the reproductive system using controlled conditions, have identified the ovaries, uterus, and oviduct as targets of cigarette smoke (Shiverick and Salafia, 1999, Talbot, 2008, Neal, et al., 2007, Mlynarcikova, et al., 2005, Talbot and Riveles, 2005). In vitro culture of post-implantation embryos has been used to show that nicotine, a major component of tobacco smoke, retards growth in the brain and branchial arches of rats (Joschko, et al., 1991) and induces apoptosis in both the brain and spinal cord of mice (Zhao and Reece, 2005). Recently, the “embryonic stem cell test” was introduced to monitor the effects of chemicals on embryoid bodies derived from mESC (zur Nieden, et al., 2004, Seiler, et al., 2006). This assay, which models post-implantation development, is a good predictor of chemicals that are teratogenic. In the current study, mESC were used as a model to test the toxicity of cigarette smoke on pre-implantation development.
Most prior work on cigarette smoke has been done using either research (e.g., 2R1 or 1R4F) or traditional (full-flavored) commercial (e.g., Marlboro Reds) brands of cigarettes. 2R1 (high-tar unfiltered) and 1R4F (lower tar and filtered) research cigarettes were manufactured by the University of Kentucky to provide researchers with standardized cigarettes for testing. In addition to traditional brands such as Marlboro Reds, cigarette companies now market “harm reduction” brands (e.g., Marlboro Lights, Advance Lights, and Quest) which claim to have reduced levels of toxicants, in particular carcinogens. Harm reduction cigarettes are manufactured by incorporating ventilation holes into filters to dilute smoke before it is inhaled (e.g., Marlboro Lights), using alternate curing processes to reduce tobacco specific nitrosamines (e.g. Advance Lights), adding chemicals such as palladium to the tobacco leaves to reduce tobacco-specific nitrosamines and polycyclic aromatic hydrocarbons, both carcinogens (e.g. Omni), and genetically engineering the tobacco plant to significantly reduce nicotine concentration (e.g., Quest) (http://www.apa.org/science/psa/sb-hatsukami.html). Cigarette companies have generally claimed that harm reduction cigarettes are not as dangerous to smokers’ health as traditional brands (http://www.apa.org/science/psa/sb-hatsukami.html). However, it is now known that harm is not reduced in the case of “light” or “low yield” cigarettes. To obtain adequate concentrations of nicotine, smokers of “light” cigarettes (a type of harm reduction product) inhale larger puffs, smoke more cigarettes, and smoke down close to the butt thereby making their exposure
equivalent to or greater than smokers of traditional “full flavored” cigarettes (Warner, 2005).

Compared to traditional and research brands, there are relatively few studies on harm reduction cigarettes and their effects on reproduction. It has been shown that MS and SS smoke from both traditional and harm reduction cigarette smoke inhibit ciliary beat frequency, oocyte pick-up rate, and smooth muscle contractions of the hamster oviduct (Riveles, et al., 2007). These data indicate that while harm reduction cigarettes may have reduced the levels of carcinogens, MS and SS smoke from these products still retain toxicants that are inhibitory in diverse biological assays involving the oviduct. The purpose of this study was to compare the toxicity of MS and SS smoke from traditional and harm reduction cigarettes using embryonic stem cells as a model for pre-implantation embryos.
2. Materials and Methods

Chemicals and Tissue Culture Supplies

Dulbecco’s Modified Eagles Medium (DMEM), penicillin/streptomycin, L-glutamine, and β-mercaptoethanol were purchased from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), 100 mM sodium pyruvate, and 1X trypsin/EDTA were purchased from Gibco (InVitrogen, Carlsbad, CA, USA). Non-essential amino acids were purchased from ATCC (Manassas, VA, USA). Leukemia inhibitory factor (LIF) was purchased from Chemicon International (Temecula, CA, USA). Tissue culture flasks (T-25 and T-75) were from Nunc (Fisher Scientific, Tustin, CA, USA). Tissue culture plates (35x100 mm) were from Falcon (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Phosphate buffered saline (PBS) was made using deionized water, autoclaved, and stored at 4°C. Research cigarettes (2R1 and 1R4F) were purchased from the University of Kentucky. Commercial brand cigarettes were purchased from retail dealers, and these included Marlboro Red (filter cigarettes, tar = 15 mg, nicotine = 1.1 mg) and Marlboro Lights (filter cigarettes, tar = 10 mg, nicotine = 0.8 mg) from Philip Morris Inc. (Richmond, VA, USA); Advance Premium Lights 100s (filter cigarettes, tar = 10 mg and nicotine = 0.8 mg) from Brown and Williamson Tobacco (Louisville, KY, USA); Quest (filter cigarettes, tar = 10 mg and nicotine ≤ 0.05 mg) from Vector Tobacco Inc. (Mebane, NC, USA).
Preparation of Smoke Solutions

MS and SS smoke solutions were prepared using a University of Kentucky analytical smoking machine. MS smoke solutions contain tobacco toxicants inhaled by active smokers whereas SS smoke solutions contain chemicals inhaled by passive smokers. Both MS and SS smoke solutions were made in DMEM medium, and 10 cigarettes were used to achieve the concentration of 100 puffs of smoke dissolved in 5ml of medium (10 puffs/cigarette). Concentrations of smoke solution were measured in puff equivalents (PE). PE is defined as the number of puffs of cigarette smoke dissolved in 1ml of aqueous solution; (1 PE = the smoke from 1 puff that dissolves in 1 ml of medium). Serial dilutions were performed to achieve the PEs desired. All experiments were done using 0.0PE (control), 0.01PE, 0.1PE, or 1.0PE of either MS or SS smoke from research, traditional (Marlboro Red) or harm reduction (Marlboro Lights, Advance Lights, and Quest) cigarettes. In some experiments, MS smoke solutions were made after removing the filter from cigarettes.

Cell Cultures

Mouse embryonic fibroblasts (mEFs) were isolated from 13.5 day old embryos using the American Type Culture Collection (ATCC) protocol. Fibroblast cultures were expanded on 0.2% gelatin coated Nunc T-25 flasks (Fisher Scientific, Tustin, PA, USA) and irradiated with cesium$^{137}$ (8,000 rads for 126.25 minutes) at passage 3 when cultures were 90-95% confluent. mEF culture medium was
changed to regular embryonic stem cell medium for at least 1 hour prior to stem cell plating.

D3 mESC were purchased from the ATCC (CRL-11632 from ATCC, Manassas, VA, USA). All experiments were done with passages 9-24. D3 mESC were plated on mitotically inactivated mEFs in stem cell medium containing 81.5% DMEM, 15% FBS, 0.98% L-glutamine, 0.98% sodium pyruvate, 0.98% non-essential amino acids, 0.5% penicillin/streptomycin, 0.00065% β-mercaptoethanol, and 0.00025% LIF. The medium was changed daily, and cell confluency was also examined. Cells were used for experimentation or frozen down at 70-75% confluency. Stem cell cultures were used in experiments 48-72 hours after plating. All cultures were maintained in a 37°C, 5% CO₂ incubator.

**Animals**

NIH Swiss white mice, purchased from Harlan (San Diego, CA, USA), were housed in the University of California, Riverside vivarium. The mice were on a 14-hour light and 10-hour dark cycle, and they were fed Purina rodent chow (Ralston-Purina, St. Louis, MO). Animal protocols were approved by the Campus Committee on Animal Care.

**Collection of Pre-implantation Embryos**

For collection of pre-implantation embryos, NIH Swiss white mice were superovulated and mated. To induce superovulation, mice were injected intraperitoneally with 10 IU of PMSG at 1430 hours followed by 10 IU of HCG after
46 hours. The female mice were then placed in cages containing two male mice. Pre-implantation embryos were collected 3 days after mating by flushing the oviducts with mESC medium.

**mESC Attachment, Survival, and Proliferation Assay**

To examine the effects of cigarette smoke on mESC attachment, survival, and proliferation, experiments were done over 24 hours. Mouse ESCs on mEF feeder layers were detached using 0.05% trypsin. In each experiment, mESCs were separated from fibroblasts by plating on 0.2% gelatin coated 60mm dishes. After 25 minutes, the supernatant containing mainly mESC was collected, and the procedure was repeated again. After mESC isolation, the number of cells needed for each sample was determined using a hemacytometer. Tissue culture dishes (35mm) were coated with gelatin, and mESCs were plated at 100,000 cells per dish in medium containing varying doses of smoke solutions. Images were taken at 0, 6, and 24 hours, and the number of attached cells was determined at 6 and 24 hours.

**Effect of Smoke Solutions on Pre-attached mESC**

To determine the effect of smoke solutions on pre-attached mESC, cells were plated on gelatin coated dishes (100,000 cells per dish) for 6 hours to allow maximum attachment. Cells were then treated with Advance MS or SS smoke (0.0PE, 0.01PE, 0.1PE, or 1.0PE) for 24 hours, at which time the number of attached cells was counted.
**Pre-treatment of ESCs With Cigarette Smoke**

ESCs, separated from the fibroblasts, were incubated in 0.0PE or 1.0PE of Advance MS or SS smoke solution for 1 hour in low attachment dishes (Corning Inc., Corning, NY, USA) at a density of 100,000 cells per plate. After treatment, stem cells were collected, centrifuged, resuspended in fresh medium without smoke solution, and re-plated on 0.2% gelatin coated 35mm dishes in medium that did not contain smoke solutions. Images and the number of attached cells were recorded at 6 and 24 hours.

**Apoptosis Detection Assay**

To determine if cigarette smoke induced apoptosis in mESC and pre-implantation embryos, FLICA Caspase Detection Kits (Immunochemistry Technologies, LLC, Bloomington, MN, USA) were used to stain mESC or pre-implantation embryos for activated caspases. mESC were treated with MS or SS cigarette smoke for 6 hours, then incubated in the polycaspase-FLICA™ for 30 minutes to detect polycaspase activity. Pre-implantation embryos were incubated in MS or SS smoke solution for 1 hour, then treated with SR-DEVD-FMK Caspase 3&7 FLICA™ reagent for 30 minutes. After staining with FLICA reagents, cells or embryos were washed 3 times for 20 minutes using the washing buffer provided with the kit, then samples were mounted on glass slides, and viewed using a Nikon fluorescent microscope.
Statistical Analysis

Statistical significance was evaluated using a one sample $t$-test to compare the control group (untreated), which was set to 100%, with the mean percent of each treatment group for each endpoint assay. GraphPad (GraphPad, San Diego, CA, USA) was used for all statistical analyses. Means were considered to be significantly different for $p < 0.05$. 
3. Results

Basis for mESC Attachment Assay

The purpose of the first experiment was to determine the optimal time for quantifying mESC attachment to 0.2% gelatin. Preliminary trials suggested that mESC attached during the first 6 hours of incubation (not shown). To test this more rigorously, cells in control medium or in various doses of MS or SS smoke solution were plated, and the number of suspended cells was counted at 6, 12, and 24 hours. The number of suspended cells in both MS (Fig. 3.1) and SS (not shown) smoke solution leveled off after 6 hours in all groups indicating that attachment was complete by this time. In subsequent experiments, attachment was compared at 6 hours, the time of maximum attachment.

MS and SS Smoke Solutions from Traditional and Harm Reduction Cigarettes

Inhibited mESC Attachment Dose Dependently
Traditional (Marlboro Red) and harm reduction (Marlboro Lights, Advance, and Quest) MS and SS cigarette smoke solutions were tested for their effects on mESC attachment at 6 hours using 3 doses (0.1, 0.01, and 1.0PE). For each cigarette brand, the number of attached cells was plotted relative to the control which did not contain smoke solution (Fig 3.2 A-D). MS (filtered and non-filtered) and SS smoke

![Graphs showing the effects of smoke on mESC attachment]
solutions from traditional and harm reduction cigarettes significantly inhibited mESC attachment dose dependently. In all four brands of cigarettes, non-filtered MS smoke was more inhibitory than filtered MS smoke, showing that the filter removed toxicants. In general, SS smoke and non-filtered MS smoke had similar inhibitory activity. Attachment was also inhibited dose dependently by MS and SS smoke from 2R1 and 1R4F research brand cigarettes (not shown).

**Basis for Evaluating Death, Survival, or Proliferation of Smoke Treated mESC**

To determine if attached mESC die, survive, or proliferate in the presence of smoke solutions, the number of attached cells was counted at 24 hours and compared to values at 6 hours. Three possible outcomes were observed as shown in Figure 3.3. Relative to the number of attached cells at 6 hours, cell number at 24 hours either increased (proliferation occurred), stayed the same (cells survived), or decreased (cells that had been attached died).
**MS and SS Smoke Inhibited mESC Proliferation and Survival Dose Dependently**

Filtered MS smoke solutions from the four brands of cigarettes were tested for their effects on mESC proliferation (Fig. 3.4A-D). Filtered MS smoke from all brands of harm reduction cigarettes significantly inhibited proliferation dose dependently. In contrast, filtered MS smoke from Marlboro Red traditional cigarettes did not have a

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**Fig. 3.4** Filtered MS smoke inhibited survival and proliferation of mESC dose dependently. (A) Marlboro Red filtered MS smoke, (B) Marlboro Lights filtered MS smoke, (C) Advance Lights filtered MS smoke, and (D) Quest filtered MS smoke. Each point is the mean ± standard deviation of 3 experiments. Statistical significance was determined using a one-sample t-test (*P < 0.05, **P < 0.01 and ***P < 0.001).
significant effect at any dose. At 1.0PE, the highest dose tested, MS smoke from the harm reduction brands either caused cell death (Marlboro Lights) and or enabled survival without significant proliferation (Advance and Quest). MS smoke from 2R1 and 1R4F research cigarettes produced results similar to Quest cigarettes, but were both somewhat more potent at the 0.1PE dose (not shown).

When similar experiments were done using cigarettes from which the filter had been removed, non-filtered MS smoke from all four brands had similar effects
on mESC survival and proliferation. The highest dose (1.0PE) of smoke from each brand killed all cells (Fig. 3.5A-D). At 0.1PE, cells were able to survive, but did not proliferate significantly. All non-filtered MS smoke solutions (Fig. 3.5) were more potent than filtered MS smoke (Fig. 3.4).

SS smoke solutions from all four cigarette brands showed detrimental effects on mESC survival and proliferation (Fig. 3.6A-D). At 1.0PE, cells did not survive in any treatment groups. At 0.1 PE, cells died in SS smoke from Marlboro Light
cigarettes, survived in smoke from Quest, and proliferated at a slower rate than the control in Marlboro Red and Advance SS smoke. In general, SS smoke and non-filtered MS smoke were similar in their effects on mESC survival and proliferation, while filtered MS smoke was the least potent of the three types of smoke tested. SS smoke from 2R1 and 1R4F research cigarettes produced results similar to Marlboro Lights (not shown).

To demonstrate that the higher doses of smoke induced apoptosis in mESC, cells were labeled with an inhibitor that fluoresces red when bound to activated caspases (polycaspase-FLICA). After 6 hours of incubation, 93% of the attached cells treated with 1.0 PE of SS smoke solution from 2R1 cigarettes were labeled with activated polycaspase inhibitor, while only 1% of the cells were apoptotic in the untreated control.

**Smoke Treatment Caused Loss of Pre-Attached Cells**

In all the previous experiments, mESC were plated in the presence of smoke solutions. The purpose of this experiment was to determine if cells that had attached prior to MS or SS smoke treatment would be protected from the effects of smoke solutions. mESC were plated on gelatin coated dishes in standard mESC medium, and attachment was allowed to occur. At 6 hours, stem cells were treated with different doses of Advance MS (filtered) or SS smoke, and the number of attached cells in each group was counted at 24 hours. In both MS and SS groups, the number of pre-attached cells decreased significantly with increasing smoke
concentrations (Fig. 3.7A-B). Moreover, all attached cells in 1.0PE of SS smoke were detached from the plates after 24 hours of treatment. In agreement with previous experiments, SS smoke was more potent than MS smoke for all doses.

**Smoke Pre-Treatment of mESC Inhibited Attachment and Proliferation**

![Graph](image)

Fig. 3.8 Pre-treatment of mESCs with Advance MS (filtered) (A) or SS (B) smoke solutions before plating inhibited attachment (MS and SS), survival (SS), and proliferation (MS). Each point is the mean ± standard deviation of three experiments. Statistical significance was determined using a t-test at 6 hours and at 24 hours (*P < 0.05, **P < 0.01 and ***P < 0.001).
To determine if mESC can be rescued from smoke solution treatment, cells were pre-treated with Advance MS (filtered) or SS smoke for 1 hour in low attachment dishes then re-plated onto gelatin coated dishes with fresh medium that did not contain smoke solution (Fig. 3.8A-B). At 6 and 24 hours, the number of attached cells was determined. Pretreatment of mESC with Advance MS or SS smoke solutions significantly inhibited attachment at 6 hours. At 24 hours, attached cells in MS smoke solutions were able to proliferate, but at a slower rate than in the control group. In SS smoke solution, the number of attached cells at 24 hours decreased slightly when compared to the 6 hour group.

Smoke Treatment Induced Apoptosis in Pre-implantation Mouse Embryos

Since mESC represent a proxy for the inner cell mass of pre-implantation embryos, the effect of smoke solutions on actual mouse embryos was tested. Pre-implantation embryos were treated with 0.1PE of Advance MS or SS smoke for 1 hour then stained with a FLICA reagent that detects activation of caspases 3&7. In smoke treated embryos, some blastomeres were morphologically abnormal and appeared to be degenerating compared to the control (Fig. 3.9A-C). In addition, blastomeres in smoke treated embryos were positive for activated caspases 3&7, while control blastomeres were negative (Fig. 3.9 D-F).
Fig. 3.9 Smoke treatment induced apoptosis in pre-implantation embryos through activation of caspases 3 and 7. (A-C) Hoffman contrast images of pre-implantation embryos incubated in control medium (A), 0.1PE of Advance MS smoke solution (B), or 0.1PE of Advance SS smoke solution (C). (D-F) The same pre-implantation embryos viewed with fluorescence microscopy to visualize activated caspases 3 & 7. (D) Absence of activated caspases 3 and 7 in blastomeres of control untreated embryo, (E) Presence of activated caspases 3 and 7 in embryo treated with Advance filtered MS smoke, (F) Detection of activated caspases 3 and 7 in embryo treated with SS smoke.
4. Discussion

We have developed and used a straightforward rapid \textit{in vitro} assay based on attachment, proliferation, survival, and death of mESC to compare the toxicity of traditional and harm reduction tobacco smoke. Because mESC originate from the inner cell mass of blastocysts, this assay models the effects of smoke on pre-implantation development. For all endpoints (cell attachment to a gelatin, survival, proliferation, and death), harm reduction cigarette smoke (Marlboro Lights, Advance Lights, and Quest) was as potent as or more potent than traditional cigarette smoke (Marlboro Red). In addition, our data showed that for all brands tested, SS smoke solutions were considerably more potent than filtered MS smoke solutions. However, removal of the filter increased the toxicity of MS smoke to the levels observed for SS smoke. Pre-attachment of cells to gelatin did not protect them from the effects of MS or SS smoke solutions. Moreover, pretreatment of cells with smoke solution for only 1 hour before plating was sufficient to reduce attachment, proliferation, and survival when cells were subsequently plated in medium that did not contain smoke solution, indicating that relatively short exposures to smoke are sufficient to induce harm in mESC and that harm was not readily reversible. Mouse pre-implantation embryos behaved similarly to mESC when exposed to smoke solutions, supporting the idea that mESC are a valid model for pre-implantation embryos. These data are consistent with the idea that cigarette smoke is toxic to pre-implantation embryos and can retard growth or kill embryonic cells at this stage of development.
Attachment of cells to each other and to extracellular matrices is necessary for normal embryonic development. When mESC were plated in the presence of smoke solutions, attachment to gelatin was inhibited by both MS and SS smoke from each brand of cigarette tested. Pre-attachment of cells to gelatin did not protect them from smoke treatment. Pre-attached cells underwent detachment upon exposure to either MS or SS smoke. The magnitude of this effect was very similar in terms of potency whether cells were attached first or treated with smoke during attachment (compare Figs. 3.4 and 3.6 with Fig. 3.7). The attachment of human ESC to Matrigel is inhibited by nicotine, a major constituent of tobacco smoke (Zdravkovic, et al., 2008). In our studies Quest, which does not contain nicotine, was effective at inhibiting attachment of mESC to gelatin suggesting that in complex mixtures of smoke more than one molecule is involved in blocking attachment. Similar interference of smoke with attachment of both undifferentiated and differentiated cells has been observed in other systems. In humans, cigarette smoking down regulates the I-selectin adhesion system in the placenta, an effect that appears to be mediated by nicotine (Zdravkovic, et al., 2006). Similarly, differentiated bovine bronchial epithelial cell attachment to fibronectin in vitro is inhibited by short term exposure to cigarette smoke condensate (Cantral, et al., 1995).

Proliferation and growth are among the most important processes in prenatal development. At no other time does mitosis occur at such a high rate in mammals. Both MS and SS smoke solutions from traditional and harm reduction
cigarettes inhibited proliferation (lower doses) and survival (higher doses) of mESC. At high doses, apoptosis was induced through caspase activation. If toxicants in smoke similarly decrease proliferation and/or survival of cells in preimplantation embryos of human smokers, the consequences could be very significant. Possible outcomes could include reduced birth weight, as has been reported in offspring of women who smoke (Andres and Day, 2000, Hruba and Kachlik, 2000, Steyn, et al., 2006), reduced populations of stem and progenitor cells that would be necessary for subsequent growth and organ development, or development of congenital defects due to loss of normal cell numbers. Defects due to increased apoptosis have been observed in explanted mouse embryos cultured in the presence of nicotine (Zhao and Reece, 2005), and digit defects have been reported to increase in offspring of women who smoke (Man and Chang, 2006).

It is interesting that smoke treatment of mESC, in most cases, did not kill all mESC. In most treatment groups, even at the highest doses, some cells survived, and at lower doses, even proliferated, albeit at slower rates than the control. Therefore smoke exposure may not outright kill all cells in preimplantation embryos, but may reduce cell numbers by inhibiting division or killing a fraction of the cell population. Indeed, in the actual pre-implantation mouse embryos that we examined, not all blastomeres were killed by smoke treatment. This interesting result suggests that there is a population of cells within the mESC population, and by extension within the inner cell mass the pre-implantation embryo, that is more sensitive to smoke treatment than the surviving cells. It would be interesting to know if the cells that
are killed are in some way developmentally distinct from those that survive and growth normally.

When mESC were pretreated for only 1 hour with smoke solutions, they likewise were inhibited from attaching and as seen in the prior experiments, survival of those that attached was impaired. The magnitude of these effects was greater for SS smoke than for MS smoke. These data indicate that the action of smoke on the mESC occurs relatively quickly, and in this experimental design was not readily reversible. If similar effects occur in women who smoke, damage could be done to the pre-implantation embryo well before the smoker knew that she was pregnant.

Our data reveal several important points about the types of cigarette smoke that were tested with mESC. As we have reported previously using other assays (Riveles, Tran, Roza, Kwan and Talbot, 2007, Gieseke and Talbot, 2005, Melkonian, et al., 2002, Melkonian, et al., 2000, Knoll and Talbot, 1998), SS smoke from all brands of cigarettes was consistently more toxic than MS smoke in the mESC assays. SS smoke contains higher levels of potential toxicants than MS smoke which is produced by burning tobacco at a higher temperature (Riveles, Tran, Roza, Kwan and Talbot, 2007, EPA, 1992). In our study, SS smoke solutions were compared to MS smoke on a PE basis. In an actual smoking situation, SS smoke would be diluted in air before being inhaled by an active or passive smoker, and this dilution would tend to benefit the smoker. However, levels of SS smoke in some passive smoking environments can be high (e.g. in an enclosed automobile or smoky bar) and
exposure may occur for relatively long periods of time. Our data indicate that such exposures may not be safe for pregnant women with pre-implantation embryos.

When comparing filtered and non-filtered MS smoke, the presence of a filter on the cigarette greatly reduced the potency of MS smoke, although the filter was not sufficient to completely eliminate toxicity. While filters are generally thought to remove toxicants, our data specifically show that mESC and by extension pre-implantation embryos benefit by filtering MS smoke before exposure to it. Since SS smoke can not be filtered, there is not a clear simple way to reduce the toxicants released into the environment from this type of smoke. Moreover, active smokers who use filtered cigarettes will also be exposed to their own SS smoke which in combination with their inhaled MS smoke may pose a threat to pre-implantation embryos.

Our data show that, in the mESC assay, MS and SS smoke solutions from harm reduction cigarettes are as potent or more potent than smoke from a traditional brand (Marlboro Red). We previously showed that smoke from harm reduction cigarettes impaired functioning of the hamster oviduct in ciliary beat frequency, oocyte pick-up rate and muscle contraction assays (Riveles, Tran, Roza, Kwan and Talbot, 2007, EPA, 1992). In general, in both studies, SS smoke was more potent than MS smoke. However, the mESC were overall more sensitive to lower doses of harm reduction smoke than the oviduct, supporting the idea that developing tissues are more severely affected by environmental toxicants than adult tissues (Grandjean et al., 2007), and demonstrating the importance of assessing toxicity on prenatal
stages of development. Harm reduction cigarettes are marketed as having lower levels of carcinogens and accordingly being safer to smoke (Warner, 2005). This can give smokers a false sense of security as they think that harm reduction brands lower their exposure to toxicants (Shiffman, et al., 2001, Hamilton, et al., 2004, Pederson and DE, 2007). However, it has never been shown that harm is in fact reduced when using these products (Pankow, et al., 2007), and our tests with harm reduction smoke clearly show this type of smoke retains toxicity. In the mESC assays, toxicants that could affect prenatal development were still present in filtered MS smoke from harm reduction brands, while SS smoke, which is not filterable, was highly toxic.

Finally, embryos and fetuses are more sensitive to toxicants than adults, and it is important to adjust acceptable levels of exposure to toxicants to levels that are not damaging in utero (Grandjean et al., 2007). New in vitro assays are needed that can be used to screen the effects of environmental chemicals on various stages of prenatal development. It is particularly important to have assays that monitor different prenatal stages since time of exposure to toxicants is as important dose. Other assays have been developed to monitor later stages in development using explants of rodent embryos (e.g., Joschko, Dreosti and Tulsi, 1991, Zhao and Reece, 2005) and using embryonic stem cells formed in to embryoid bodies that model post-implantation development (Seiler et al., 2006). The assay that we used in this study provides a simple, quick method for accessing environmental toxicants on mESC, a model for the inner cell mass of pre-implantation embryos. This assay could
be used to screen any chemical for toxicity on this stage of development. Given the high percent of embryos that never implant or that spontaneously abort within two weeks of implantation, it is likely that the preimplantation stages of development, which occur before a woman knows that she is pregnant, are very critical and important to study. We are currently adapting our assay to human ESC which will give more direct information on our species.

In conclusion, mESC provide a rapid assay for modeling the effects of environmental toxicants, such as tobacco smoke, on pre-implantation stages of development. By using mESC, animal usage can be minimized, and data can be obtained on important developmental parameters within a 24 hour period. Moreover, the murine model provides easy access to resources, a rapid straightforward assay, and minimizes ethical controversies. This study confirms the effectiveness of using ESC as a novel model to study embryo toxicity during pre-implantation development. Further work is being directed at developing human ESC for similar testing. Using mESC to evaluate cigarette smoke enabled relative comparisons between the toxicity of different types of smoke from different brands of cigarettes. Our data confirm that on a per puff basis, SS smoke is more toxic than MS smoke and that smoke from harm reduction cigarettes is as potent or more potent than smoke from a traditional brand.
5. References


CHAPTER 4

VIDEO BIOINFORMATICS ANALYSIS OF HUMAN EMBRYONIC STEM CELL COLONY GROWTH
Abstract
Because video data are complex and are comprised of many images, mining information from video material is difficult to do without the aid of computer software. Video bioinformatics is a powerful quantitative approach for extracting spatio-temporal data from video images using computer software to perform dating mining and analysis. In this article, we introduce a video bioinformatics method for quantifying the growth of human embryonic stem cells (hESC) by analyzing time-lapse videos collected in a Nikon BioStation CT incubator equipped with a camera for video imaging. In our experiments, hESC colonies that were attached to Matrigel were filmed for 48 hours in the BioStation CT. To determine the rate of growth of these colonies, recipes were developed using CL-Quant software which enables users to extract various types of data from video images. To accurately evaluate colony growth, three recipes were created. The first segmented the image into the colony and background, the second enhanced the image to define colonies throughout the video sequence accurately, and the third measured the number of pixels in the colony over time. The three recipes were run in sequence on video data collected in a BioStation CT to analyze the rate of growth of individual hESC colonies over 48 hours. To verify the truthfulness of the CL-Quant recipes, the same data were analyzed manually using Adobe Photoshop software. When the data obtained using the CL-Quant recipes and Photoshop were compared, results were virtually identical, indicating the CL-Quant recipes were truthful. The method described here could be applied to any video data to measure growth rates of hESC or other cells.
that grow in colonies. In addition, other video bioinformatics recipes can be
developed in the future for other cell processes such as migration, apoptosis, and
cell adhesion.
1. Materials

- mTeSR®1 Human Embryonic Stem Cell Maintenance Medium (Stem Cell Technologies, Vancouver, Canada, Catalog #05850) or any suitable medium for hESC culture.
- BD Matrigel (BD Bioscience, San Jose, CA, Catalog #356234) or other suitable substrate.
- DMEM/F12 Basal Medium (Invitrogen, Carlsbad, CA, Catalog #11330-032)
- Phosphate Buffered Saline without Ca\(^{2+}\) and Mg\(^{2+}\)
- Accutase\(^{TM}\) Enzyme Cell Detachment Medium (eBioscience, San Diego, CA, Catalog #00-4555-56) or other suitable detachment enzyme.
- 3mm Glass beads (Fisher Scientific, Catalog #11-312A), optional.
- 12-well Tissue Culture Plates (BD Falcon, San Jose, CA, Catalog #353043) or any other plate format.
- Nikon BioStation CT/IM (or other incubator/microscope suitable for collecting video data)
- CL-Quant software (Nikon) and/or Photoshop (Adobe).
2. Protocol

Part 1: Experimental Procedure:

Video data contain an abundance of information. However, this information is often difficult to extract and when done manually by humans and may require many hours of personnel time to complete. Manual analysis by humans is also subject to variation in interpretation and error. Video bioinformatics involves the use of computer software to mine specific data from video images. This method of analysis is rapid and can eliminate error that occurs when analysis is done manually by humans. The purpose of this article is to demonstrate a method for quantifying human embryonic stem cell colony growth using a video bioinformatics method.

In this paper, time lapse video images were collected using a Nikon BioStation CT incubation unit that allows multiple fields of cells to be imaged over time (Fig. 4.1). The methods described in this report are applicable to video data collected by any video microscopic set up.

![Fig. 4.1 Film strip of a hESC colony showing frames at various times during 48 hours of growth in a BioStation CT. Frames were taken at 7 minute intervals.](image)

In our experimental design, we plated H9 hESC in 12-well tissue culture plates for 48 hours. During this interval, colonies were allowed to fully attach and spread on Matrigel. Then, plates with attached hESC colonies were transferred to the BioStation CT and incubated for an additional 48 hours. While in the BioStation,
images of the colonies were collected at 7 minute intervals and were later used to create time-lapse video sequences. Videos for each colony were then analyzed to quantify colony growth using video bioinformatics recipes developed with the CL-Quant software. The analyses done using recipes created with the CL-Quant software were checked for truthfulness manually using Adobe Photoshop.

**Part 2: Preparation of attached hESC colonies:**

- Grow hESC on Matrigel coated 6-well plates, and once 70% confluency is reached, replate one well of the 6-well plate into 5 wells of a 12-well Matrigel coated plate as follows.
- Aspirate medium from a well containing hESC.
- Rinse well 2x with 1ml of PBS.
- Add 1ml of Accutase and incubate for 1 minute at 37°C and 5% CO₂.
- Add 10-12 glass beads into the well and shake the plate gently until colonies completely detach from the bottom of the well.
- Neutralize Accutase using 1ml of mTeSR medium or mEF conditioned medium.
- Centrifuge the cell suspension in a 15ml conical tube at 200g for 3 minutes.
- Decant the supernatant and the break pellet with 500μl of fresh mTeSR medium.
- Plate drop wise 100μl of the hESC suspension into each well in a 12-well plate.
• Rock plate back and forth gently and observe cultures under a light microscope. Make sure that cell clumps are evenly distributed throughout the plate.

• Place the 12-well plate back into the incubator.

• Allow hESC to attach and grow for 48 hours (a shorter time may be used).

• After 48 hours, decant the medium and wash wells with 500ul of PBS to remove unattached cells.

• Add 1ml of mTeSR medium to each well.

• Immediately place the plate into the incubator/microscope for video imaging.

• Begin collecting time-lapse images. Try to select fields with discrete single colonies that are not likely to grow into other colonies.

• Other methods for culturing and setting up hESC can be used in place of the above protocol.

Part 3: Video Bioinformatics:

CL-Quant software was used to create three “recipes” that when run in sequence will determine the pixel number or area in microns for each colony over time. The three recipes are built in sequence and for this application include segmentation, enhancement, and measurement.

Analyzing hESC colony growth using the CL-Quant software/recipe development:
• The segmentation recipe is created first.
  • Scan the entire video to make sure that the video is usable. Check to be sure the entire colony remains in the field of view and that other colonies do not enter the field.
  • Open the segmentation wizard and click the “next” button.
  • Select the correct image channel (i.e., phase, green, red, etc) and click the “next” button.
  • Pick “soft matching” out of the 3 choices and click “next”.
  • Select “want” regions by circling regions on the image—typically the outer edge of the colony to the central area of the colony.
    o This area should be as small as possible but should be representative of the entire colony.
    o When using phase contrast microscopy, be sure to include the halo around the colony for precise colony selection by the software.
    o Pick 1 or 2 regions of interest that may show different pixel patterns within the colony, but do not pick too many “wants” which may result in inaccurate mask application.
  • Click the “next” button.
  • Select “don’t want” regions by circling regions on the image that are not part of the colony and not part of the background. These are
regions that have patterns you wish to suppress and include pixels of artifacts such as debris and dead cells. Click “next”.

- Select “background” by circling regions you wish to suppress.
  - The “don’t want” and “background” regions differ in that background should be uniform and likely to show up in every frame.
  - Typically we select the grey background around the colony.

- Click the “next” button.

- A colored mask should be displayed over your region of interest (Fig 4.2A).

![Fig. 4.2](A) Image of a hESC with a mask placed on the colony by the segmentation recipe. Areas of debris and background are also masked indicating that an enhancement recipe is needed to improve the accuracy of the evaluation. (B) Image of the same colony as shown in “A” after enhancement has been performed. The mask now selects only the colony and noise has been eliminated from the selection.

- If the mask does not accurately cover the region of interest, the segmentation threshold range (located at the bottom right hand corner of the software display) may be increased or decreased.
• The segmentation wizard prompts for further mask area fine tuning if necessary.

• If you wish to change your mask, select “update soft matching regions”. This allows you to change the “want”, “don’t want”, or “background” areas.

• If the mask is satisfactory, select “apply threshold and save my mask”, and click the “next” button.

• The mask will then be displayed in a different color.

• Select “finish”.

• The recipe should be displayed on the right upper corner of the software, and it should be called “segmentation recipe”.

• Rename the recipe if you wish by right clicking and “rename”.

• For spot checking, right click and place the mouse over “apply recipe”.

• A menu will show that allows you to select the number of frames you wish use when spot checking the recipe. Run the recipe for every 10 frames to spot check the accuracy of mask placement.

• If the mask picks up parts of small unwanted regions or debris, you can create an “enhancement recipe” to improve fitting of the mask to the region of interest.
• To create an enhancement recipe, right click the “enhancement recipe” folder, and then click “new”. A new enhancement recipe should be displayed.

• From the original field of view (FOV), click the “toggles enhancement module” button located to the right of the image. Mouse over icons in the toolbar to identify the correct one.

• Select “labeling” dropdown menu.

• Select “labeling 4 connected”.

• Select “labeling 4 connected2”.

• A new task bar should be displayed.

• Grab initial mask (mask0) from the segmentation recipe and drag it to the “input” box.

• The bar should now display “input mask #”.

• Drag the icon with the “input mask #” to the mask 0 icon.

• Find “min size” in task bar and adjust number until only region of interest is displayed. Make sure you click on “execute” through each fitting trial.

• Once you are satisfied with your minimum size adjustment, click on the “enhancement recipe” you’ve created previously. (Fig. 4.2B)

• At the bottom of the screen, select “save to recipe”.

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• The software now prompts to “overwrite selected recipe?”, select “yes”
• Spot check the enhancement recipe using the same procedures as above for spot checking segmentation.
• If satisfied with the segmentation and enhancement recipes, select ‘create measurement template’ on the right hand toolbar.
• Rename the template to if you wish.
• In the measurement template window, move cursor over clipart cell figure. Hold the Ctrl key and select the cell.
• Uncheck ‘default parameter’ and check ‘area parameter’ under ‘morphology’.
• Exit ‘template’ window.
• Select the icon created in the measurement recipe.
• Rename the recipe.
• Move to and select ‘template’ tab in the upper right corner.
• Drag and drop your previously created measurement template onto the measurement window.
• Click ‘Yes’ to continue.
• In the measurement recipe window, select ‘Channel mappings’, then ‘Whole cell’.
• Select ‘Mask mappings’.
• Then select ‘Whole cell’ option for enhanced mask.
• Select the 'Measurement recipe' under the Recipe tab in the main window.
• Select the ‘Save’ icon within the Measurement recipe window.
• Then close the window.
• Now, close and reopen the ‘FOV’.
• Right click the ‘Recipe’ icon, select ‘Lock recipe list’ and run the recipes sequentially on your video data.

Part 4: Checking recipe accuracy using the Photoshop software

In order to validate the recipe created with (CL-Quant software), the same data can be analyzed manually with Adobe Photoshop. For this analysis, every 10th frame (every 70 min time point) was analyzed to measure colony size over 48 hours.

• Open a frame of a colony image in Adobe Photoshop.
• Click on the Magic Wand Tool in the toolbar.
• Click on the area around the colony so the entire field is covered except the colony region.
• Click ‘Edit in Quick Mask Mode’ in the toolbar and click on the colony so the colony is selected.
• Make sure the dotted line around the colony fits right around the periphery of the colony and not inside it. If the dotted line is not around the periphery, change the tolerance value in the upper toolbar accordingly.

• Go to the ‘Window’ pull down menu and click ‘Histogram’.

• Write down the pixel value when cache=1. If cache is 2, click the ‘!’ to change to cache 1, and then note the pixel value.

• Repeat the above process for every 10th frame. Fewer total frames can be used if desired.

• Data collected using Photoshop and CL-Quant software can then be plotted together. If the CL-Quant recipes are truthful, the curves for the Photoshop and CL-Quant analysis should be very similar (Figs. 4.3-4.5).

• If the recipes developed with CL-Quant are truthful, they can be reliably applied to other video data.

• The value in using video bioinformatics in this analysis is that once the segmentation, enhancement, and measurement recipes are developed and validated, they will perform the analysis much more rapidly and more accurately than a human using Photoshop.
Fig. 4.3 Graph showing increase in colony size (in pixels) over 48 hours as determined using CL-Quant software and Photoshop. This graph plots the raw data and shows that colonies start at different sizes. It also shows that both methods of measurement are in good agreement.

Fig. 4.4 Graph showing the same data as in Figure 4.3 after normalization to show the percent increase in colony size over 48 hours. This shows that growth rates are similar regardless of starting colony size and that both measures of analysis give similar results.

Fig. 4.5 Graph showing the means for the normalized data in Figure 4.4. This graph clearly shows good agreement between the analyses done using video bioinformatics (CL-Quant software) and Photoshop and establishes that the recipe is truthful. The slight downturn in area for the Photoshop data at frame 325 is due to several videos not being included in the Photoshop analysis.
3. Discussion

Video bioinformatics is a powerful tool for rapidly extracting data from video images. Our protocol for quantifying hESC colony growth demonstrates one application of video bioinformatics to a biological problem. This method is quantitative and has the interesting feature of revealing data from individual hESC colonies. Video bioinformatics recipes can be developed to monitor other cellular processes such as proliferation, migration, apoptosis, and cell attachment to the substrate, and cell attachment to adjacent cells. The accuracy of the recipes developed using CL-Quant software were validated using Photoshop and were found to be truthful. Once the appropriate recipes are developed, video data from any source can be analyzed very quickly. The time required to analyze video data using video bioinformatics is significantly less than analysis by hand using Photoshop. Moreover, the analysis done by the computer is less prone to error as the computer will analyze the data the same way each time, while a human performing an analysis may make errors or slightly different judgments each time an image is analyzed. Although not discussed as part of this protocol, videos can also be examined for morphological changes in the colony. This parameter would be useful in cases where treatment groups are included.
CHAPTER 5

USING HUMAN EMBRYONIC STEM CELLS TO PREVENT DISEASE: COMPARISON OF TOXICITY OF SMOKE FROM CONVENTIONAL AND HARM REDUCTION CIGARETTES
**Abstract**

**BACKGROUND:** Harm reduction cigarettes are purported to be safer to smoke than conventional brands, although very little testing of this concept has been done. Our earlier study with mouse embryonic stem cells (mESC) showed that smoke from harm reduction brands was more inhibitory in attachment and growth assays than smoke from a conventional brand and that harm reduction sidestream smoke (SS) was more potent than mainstream smoke (MS). The purpose of this study was to evaluate the impact of harm reduction smoke on human embryonic stem cells (hESC) which model the earliest stages of human development.

**METHODS:** Doses of smoke were measured in puff equivalents (PE) (1 PE = the amount of smoke in one puff that dissolves in 1 ml of medium). The cytotoxic doses of MS and SS cigarette smoke for H9 hESC were determined using morphological criteria and trypan blue. Death induced by smoke occurred apoptotically, as shown by Magic Red™ staining. Attachment and proliferation of hESC were followed at a non-cytotoxic dose in time lapse videos collected using BioStation technology. Attachment and proliferation data were mined from videos either manually or using video bioinformatics recipes developed with CL Quant software.

**RESULTS:** MS and SS smoke from both conventional and harm reduction cigarettes were cytotoxic and induced apoptosis in hESC colonies at 1PE. Attachment and proliferation assays were therefore performed at 0.1PE, which was not cytotoxic. In the attachment assay, SS smoke from all four brands inhibited attachment of hESC
colonies to Matrigel with the strongest inhibition occurring in the harm reduction brands. In the assay measuring hESC colony growth, SS smoke, but not MS smoke, from all four brands was inhibitory, and two of the harm reduction brands were potent than the conventional brand. Mouse and human ESC responded similarly in most assays to smoke treatments, and, in general, human cells were more sensitive to smoke than mouse cells.

**CONCLUSIONS:** A new method based on bioinformatics analysis of time lapse video data was developed for assaying the effects of toxicants on the cellular dynamics of hESC. Data showed that SS smoke from harm reduction cigarettes is as potent as or more potent than smoke from a conventional brand. Moreover, SS smoke was more inhibitory than MS smoke in all assays. hESC appear to be better models for this type of study than mESC. Finally, results indicate that pregnant women should avoid exposure to smoke, including smoke from harm reduction products.

**Key Words:** cigarette smoke, harm reduction, embryos, humans, toxicology, tobacco, bioinformatics
1. Introduction

Tobacco smoke is comprised of both mainstream smoke (MS), which is actively inhaled by smokers, and sidestream (SS) smoke, which burns off the tip of a cigarette (EPA, 1992). SS smoke is the major component of secondhand smoke, also called environmental tobacco smoke, and is inhaled by passive smokers. Both MS and SS smoke adversely affect many reproductive processes. For example, cigarette smoke exposure decreases birth weight, while increasing the length of time to conceive, spontaneous abortions, perinatal mortality, and congenital defects (Andres and Day, 2000, Berthiller and Sasco, 2005, Higgins, 2002, Rogers, 2008, Shiverick and Salafia, 1999). In addition, in vitro assays have consistently shown interaction of cigarette smoke with female reproductive organs (Shiverick and Salafia, 1999, Mlynarcikova, et al., 2005, Talbot, 2008, Talbot and Riveles, 2005). For example, both MS and SS cigarette smoke solutions significantly impaired oviductal functioning by decreasing ciliary beat frequency, oocyte pick-up by the oviduct, and muscle contraction rates while increasing adhesion of the oocyte cumulus complex to the oviduct (Gieseke and Talbot, 2005, Riveles, et al., 2003). A similar delay in oocyte and embryo transport and muscle contraction was seen in vivo in a hamster model exposed to smoke (DiCarlantonio and Talbot, 1999). While these epidemiological studies and in vitro assays confirm that the female reproductive organs and fetuses are targets of cigarette smoke, relatively little is known about the effects of cigarette smoke on young embryos including pre-implantation stages.
In an attempt to reduce the toxicity of cigarette smoke, tobacco companies have introduced various types of harm reduction products, including harm reduction cigarettes (Warner, 2005). Harm reduction cigarettes, which are often claimed to have fewer toxins and to be less harmful than conventional brands, are made using complex filters (Marlboro Lights, Advance Lights) or by genetically altering tobacco plants to reduce nicotine concentration (Quest). In 2009, our lab developed assays based on mouse embryonic stem cells (mESC) to compare the effects of MS and SS smoke from conventional (Marlboro Red) and harm reduction (Marlboro Lights, Advance Lights, and Quest) cigarettes on attachment, survival, proliferation, and apoptosis (Lin, et al., 2009). We found that SS smoke was generally more toxic to mESC in these assays than MS smoke. We also showed, unexpectedly, that SS smoke from harm reduction cigarettes was generally more inhibitory than smoke from the conventional brand.

To fully understand the effects of smoke on human embryonic development and to avoid possible species differences in response, it is necessary to perform toxicological assays using human models. Because it is clearly not possible to directly determine chemical toxicity on actual human embryos, we have developed assays with hESC, which model pre-implantation embryos, to measure and compare the toxicity of MS and SS smoke from conventional and harm reduction products. Because hESC are more difficult to work with than mESC, we could not directly apply the methods used in our prior mouse study to hESC. We circumvented technical problems that hESC present by using BioStation technology to create time
lapse videos of cells during treatment in various *in vitro* assays (Lin, et al., 2010). By combining BioStation technology with video bioinformatics analysis (automated processing and data mining of biological spatio-temporal data), we were able to obtain quantitative data for attachment, colony growth, and survival endpoints. Our data show that: (1) hESC can be used to measure toxicity of environmental chemicals such as tobacco smoke, (2) BioStation technology coupled with video bioinformatics analysis facilitates assays with hESC, (3) SS smoke was more potent than MS smoke in all assays, and (4) SS smoke from harm reduction cigarettes was generally more potent than smoke from a conventional brand.
2. Materials and Methods

Chemicals, media, and reagents

Four commercial brands of cigarettes were purchased from retail dealers and used in this study. These included Marlboro Red (filter cigarettes, tar = 15 mg, nicotine = 1.1 mg) and Marlboro Lights (filter cigarettes, tar = 10 mg, nicotine = 0.8 mg) from Philip Morris Inc. (Richmond, VA, USA), Advance Premium Lights 100s (filter cigarettes, tar = 10 mg, nicotine = 0.8 mg) from Brown and Williamson Tobacco (Louisville, KY, USA), and Quest (filter cigarettes, tar = 10 mg, nicotine = 0.05 mg) from Vector Tobacco Inc. (Mebane, NC, USA). Marlboro Red cigarettes were chosen as they represent one of the top selling conventional brands, while the other brands are all harm reduction products.

mTeSR basal medium and mTeSR supplement (Stem Cell Technologies, Vancouver, Canada) were used to maintain hESC cultures and for experimentation. 6-well, 12-well, and 24-well plates (Falcon, Fisher Scientific, Chino, CA) were coated with Matrigel (BD Biosciences, Fisher Scientific, Chino, CA) for at least 2-3 hours at room temperature or overnight at 2-4°C. Phosphate buffered saline (PBS) was made using deionized water, autoclaved, and stored at 2-4°C. Accutase™ Enzyme Cell Detachment Medium was purchased from eBioscience (San Diego, CA, USA) and stored at -20°C.

Trypan blue (Sigma Aldrich, St. Louis, MO) solutions were made by dissolving 0.4g of trypan blue in 80ml of PBS at a low boil, cooling to room temperature, and sterilizing using 0.2µm filters (Acrodisc Syringe Filters, Pall Corporation, Ann Arbor,
MI). For the detection of apoptotic activity, the FLICA™ Poly Caspases Kit and Magic Red™ Caspase Detection Kit for caspases 3 and 7 (Immunochemistry Technologies, LLC Bloomington, MN) were used. The FLICA™ and Magic Red powder were reconstituted in DMSO (Dimethyl Sulfoxide, ATCC, Manassas, VA), aliquoted (5µl) in Eppendorf tubes, and frozen at -20°C as instructed in the manual.

**Preparation of smoke solutions**

Both MS and SS smoke solutions were prepared using an University of Kentucky smoking machine that was set up and operated as described previously (Lin, Tran and Talbot, 2009, Knoll and Talbot, 1998). Smoke solutions were made by drawing either MS or SS smoke through basal mTeSR medium without mTeSR supplement and stored at -80°C. For all experiments, different concentrations of smoke solutions were diluted in complete mTeSR medium and used immediately. One cigarette was used to achieve a concentration of 10 puffs of smoke dissolved in 5ml of medium (10 puffs/cigarette, 2 puffs/1ml of medium). Concentrations of smoke solution were measured in puff equivalents (1 PE = the amount of smoke in one puff that dissolves in 1 ml of medium). Serial dilutions of smoke solution were made to achieve the PE concentrations used for testing, which were 0.0PE, 0.01PE, 0.1PE and 0.0PE (control). Smoke solutions were made with conventional (Marlboro Red) and harm reduction (Marlboro Lights, Advance Lights, and Quest) cigarettes.
**Cell cultures**

H9 hESC, purchased from WiCell Stem Cell Institute (WI), were grown using feeder free conditions. Cells were cultured and maintained on Matrigel coated 6-well tissue culture plates in complete mTeSR medium. All cell cultures were observed daily for cell density and pluripotency, and the medium was changed everyday. Once cultures reached 60-70% confluency, hESC were removed from the plates using glass beads and Accutase without dilution (1 minute at 37°C). Once hESC began to detach, 10-12 sterile glass beads were placed into the well and rolled gently in all directions until colonies completely detached into small clumps. The cell suspension was then neutralized using mTeSR medium and centrifuged at 200x g. Colonies were resuspended into clumps of 4-5 cells for passaging and experimentation.

**Morphological cytotoxicity assay and trypan blue staining**

Colony morphology and trypan blue staining were used to evaluate the cytotoxicity of cigarette smoke on H9 hESC. Colonies of 4-5 cells were plated on Matrigel coated 24-well plates with 500μl of fresh medium for 48 hours prior to smoke treatment. After attachment, hESC colonies were treated with different concentrations of MS or SS smoke (0.01PE, 0.1PE, and 1PE), while a well of untreated colonies served as the control. Colony morphology was observed with a Nikon inverted light microscope at 6 and 24 hours of treatment, and comparisons were made between control and treated groups. After 24 hours, 50μl of 0.4%
trypan blue solution was added to each well, which contained 500μl of medium. After 5 minutes of staining, images were collected from each well. Dark blue cells were interpreted to be dying due to increased permeability of the cell membrane.

**Apoptosis detection using Poly-caspases Detection FLICA Kit and Magic Red™ Caspases 3&7 Detection Kit**

The FLICA™ Poly Caspases Kit and Magic Red™ Caspases 3&7 Detection Kit were used to identify apoptosis activity in control and smoke solution treated hESC colonies. The FLICA™ (Fluorescent-Labeled Inhibitor of Caspases) probes contain an inhibitor sequence of caspases, called VAD, linked to a green or red fluorescent probe. VAD penetrates the plasma membrane and reacts with all caspases. Active caspases inside cells covalently bind to the FLICA™ probe and thus cells appear to be fluorescent. Excess FLICA™ probe not bound to active caspases is washed out of the cells. The Magic Red™ Caspase Detection Kit contained the MR-(DEVD)₂ reagent, which was reconstituted in DMSO, stored at -20°C, and protected from light. This kit contains a cell-permeant substrate (DEVD) that is linked to cresyl violet. DEVD is specifically targeted by active caspases 3 and 7, which cause cresyl violet to fluoresce red upon cleavage of DEVD. Reagents were thawed and diluted with fresh culture media prior to usage. In our experiments, hESC colonies were pre-attached for 48 hours on Matrigel, then treated for 5 hours with smoke solution, followed by 20-25 minutes of incubation in Magic Red™. Colonies staining red were interpreted to have apoptotic enzymes (caspases 3 and 7) activated by treatment.
Use of the BioStation IM to compare the effect of smoke from conventional and harm reduction cigarettes on hESC colony attachment

The BioStation IM is a small incubation unit equipped with a microscope and camera that enables time-lapse video collection. This unit is extremely useful and efficient for studying dynamic cellular events such as cell attachment and spreading. For our experiments, adherent colonies were disassociated using Accutase and sterile glass beads. Colonies were broken up into clumps of 3-5 cells and replated onto Matrigel coated 35 mm dishes containing SS smoke at 0.1PE. All four brands of cigarettes were tested. The control contained mTeSR hESC culture medium without cigarette smoke. The dishes containing suspended cells were placed into the BioStation IM immediately, and images were taken at 1-2 minute intervals over 180 minutes. Data were analyzed at 20 minute intervals to determine which colonies were attached. Colonies were considered attached when clear spreading of the cells was observed. Videos were viewed with QuickTime, and the percentage of attached colonies was counted manually at each time point; 8-10 colonies were analyzed for each treatment and control group.

Use of BioStation CT technology to compare the effect of smoke from conventional and harm reduction cigarettes on hESC colony growth

The BioStation CT is a high-content incubation unit equipped with a microscope and camera. This innovative technology enables time-lapse video data to be collected while cells are growing in controlled incubation conditions. Videos
can then be analyzed using CL-Quant software (DR Vision, Seattle, WA) to quantify dynamic cell processes (for example colony growth). To perform experiments in the BioStation CT, hESC colonies containing 5 to 8 cells were plated on Matrigel-coated 12-well plates in mTeSR medium. After 48 hours of incubation, colonies were completely attached and spread, and the density of each well was around 30-40%. To follow colonies over time, they must be evenly distributed in the well to prevent merging. In treatment groups, medium was replaced with 500 µl of mTeSR medium containing 0.1PE of MS or SS cigarette smoke, while control wells received 500 µl of complete mTeSR medium without smoke solution. Once medium was added, the 12-well plates were placed in a Nikon BioStation CT incubator and multiple colonies in each group were imaged every 140 minutes. For each experimental and control group, 5-10 colonies of similar size were selected and followed for 48 hours. After the experiments were completed, images of hESC colonies were downloaded from the BioStation CT, and the growth of colonies was analyzed using CL-Quant software.

*Analysis of video data using video bioinformatics tools developed with CL-Quant software*

The video bioinformatics tools that were used in this study have been described in detail previously (Lin, Fonteno, Satish, Bhanu and Talbot, 2010). All video bioinformatics processing was carried out using CL-Quant Software with a custom-developed algorithm including 3 subset routines (or recipes): (1) an initial
segmentation code which utilized a channel mask to separate the colony and background in each image, (2) an enhancement code that filtered out any remaining image noise (e.g., cellular debris or foreign particulates), and (3) a measurement code that quantified the number of pixels in each colony. Data obtained with the CL-Quant analysis were plotted and analyzed to determine growth rates and percent increase in size of colonies over 48 hours. The truthfulness of the CL-Quant recipes was verified by periodically checking the fit of masks and by rerunning data to assure reproducibility.

**Statistics**

Attachment data for each brand of cigarette were analyzed using a t-test in which means of the three time points showing maximum colony attachment (80, 100, and 120 minutes) for the control and treated groups were compared for significant differences. In the colony growth assay, the means of the percentage increase in colony size at the final sampling point were compared using ANOVA. When p < 0.05 in the ANOVA, a Dunnet's post hoc test was run in which each treatment group was compared to the control. Statistical analyses were run using InStat (GraphPad, San Diego, CA.). For both the t-test and the ANOVA, p ≤ 0.05 was considered significant.
3. Results

Evaluation of Cytotoxicity of MS and SS Smoke from Conventional and Harm Reduction Cigarettes

MS and SS smoke solutions from conventional and harm reduction cigarettes were evaluated for their cytotoxic effects on hESC colony morphology (loss of cells from colonies, colony elevation, and granularity in colonies) after 6 hours of treatment with doses of 0.01PE, 0.1PE, and 1PE (Fig. 5.1; Table 5.1). Negative controls (-CN) were incubated in complete mTeSR medium (0.0PE), while positive controls (+CN) were treated with 0.5% ethanol, which induces apoptosis. Colonies in the negative control group were spread, healthy, and cobblestone-like (Fig. 5.1A). In contrast, colonies treated with ethanol showed detachment of cells and cell debris (Fig. 5.1B, arrows). MS smoke at 0.01PE (not shown) and 0.1PE did not affect hESC colony morphology or survival (Fig. 5.1C-F). However, 1PE of both Marlboro Red and Advance Lights MS smoke caused some cells to detach from the plate after 6 hours of treatment (Figs. 5.1G and I black arrows). Although colonies did not detach in 1PE of Marlboro Lights MS smoke, cells were elevated and granular, and colonies had irregular edges (Fig. 5.1H), unlike the negative control. Quest MS smoke did not affect hESC colony morphology at 1PE (Fig. 5.1J).

Like MS smoke, SS smoke solutions at 0.01PE (not shown) and 0.1PE did not affect hESC colony morphology after 6 hours of treatment (Fig. 5.1K-N). However, at 1PE, SS smoke from all four brands caused loss of cells from colonies and in some cases complete rounding up of colonies (Fig. 5.1O-R). SS smoke from Advance Lights
Fig. 5.1 Morphological assay for cytotoxicity. Colonies were incubated for 6 hours, then their morphology evaluated. (A) shows the negative control (no treatment) and (B) the positive control (0.5% ethanol). Other colonies were treated with 0.1 PE of MS smoke (C-F), 1PE of MS smoke (G-J), 0.1PE of SS smoke (K-N), or 1PE of SS smoke (O-R). Scale bars = 10μm. Arrows indicate detached cells. Representative images from three different experiments are shown.
was the most potent followed by Marlboro Red, Quest, and Marlboro Lights. Cells treated with Advance Lights SS smoke solutions completely detached from the plate and formed small round clumps (Fig. 5.1Q, black arrows). Colonies treated with Marlboro Red and Quest SS smoke solutions were partly detached (Fig. 5.1O, R, black arrows). Cells that remained attached were elevated and granular. With the morphological assay, cytotoxic effects were observed with both MS and SS smoke at 1PE, but not at lower doses, and the effects were stronger with SS smoke than with MS smoke (Table 5.1).

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<th>Morphology 6 Hours&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Trypan Blue 24 Hours&lt;sup&gt;c&lt;/sup&gt;</th>
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<td><strong>MARLBORO RED</strong></td>
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<td>MS (1PE)</td>
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<td>SS (1PE)</td>
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<td><strong>MARLBORO LIGHTS</strong></td>
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<td>MS (1PE)</td>
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<tr>
<td>SS (1PE)</td>
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<tr>
<td><strong>ADVANCE LIGHTS</strong></td>
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<tr>
<td>MS (1PE)</td>
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<td>SS (1PE)</td>
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<td><strong>QUEST (NO NICOTINE)</strong></td>
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<td>MS (1PE)</td>
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<td>SS (1PE)</td>
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Cytotoxicity was further evaluated after 24 hours of treatment with 0.01, 0.1, or 1PE by staining colonies with trypan blue (Fig. 5.2, Table 5.1). While a few control colonies had a small number of blue cells, most controls had little to no staining, indicating that cells were generally healthy and viable (Fig. 5.2A including
inset). Colonies treated with MS smoke at doses of 0.01PE (not shown) and 0.1PE (Fig. 5.2 B-E) were generally similar to the controls. However, colonies treated with 1PE of Marlboro Red or Advance Light MS smoke solutions appeared to have more blue cells than controls (Fig. 5.2F and H). SS smoke solutions at 0.01 PE (not shown) and 0.1 PE (Fig. 5.2J-M) generally produced results similar to the control. In contrast, 1PE of SS smoke solution from all brands caused colonies to detach, round up, and stain heavily with trypan blue (Fig. 5.2 N-Q), indicating the cells had died during treatment. The relative cytotoxicity of MS and SS smoke at 1PE based on the morphological and trypan blue data is summarized in Table 5.1. For all brands except Quest, MS smoke was slightly cytotoxic at 1PE (morphological assay), while SS smoke was far more cytotoxic in both assays with Advance harm reduction cigarettes being the most cytotoxic. The morphological and trypan blue data for MS and SS smoke further showed that 0.1PE was not cytotoxic for any brand.

In the cytotoxicity assays, cells in the 1PE treated colonies often exhibited blebs on their surfaces, leading us to the hypothesis that smoke-induced cell death occurred by apoptosis. This hypothesis was tested by staining for apoptosis with the Magic Red™-Caspases 3&7 Detection Kit. Control groups treated with Magic Red did not show fluorescence, indicating that the colonies were healthy and caspases 3 and 7 were not activated (Fig. 5.2 R, S). In contrast, all groups treated with 1PE of MS smoke (not shown) showed some fluorescence, while all groups treated with 1PE of SS smoke solutions were markedly fluorescent (shown for Quest in Fig. 5.2 T, U), indicating that at this dose cell death was induced apoptotically.
All subsequent experiments were done using 0.1PE of MS or SS smoke solutions since this dose was shown in the above assays to be non-cytotoxic.
Conventional and harm reduction SS cigarette smoke inhibited hESC colony attachment at a non-cytotoxic dose (0.1PE)

Our previous studies showed that cigarette smoke significantly inhibited attachment of mESC, and SS smoke was more inhibitory than MS smoke (Lin, et al., 2009). To determine if SS smoke affects attachment of hESC in a similar manner, time-lapse videos were taken of non-attached colonies using a Nikon BioStation IM, and the kinetics of attachment (percentage of attached colonies) were determined for each control and treated group. For all brands tested, SS smoke from both conventional and harm reduction cigarettes significantly inhibited colony attachment.

Fig. 5.3 Effect of 0.1PE SS smoke solutions on colony attachment in the BioStation IM. (A) control vs. Marlboro Red SS smoke, (B) control vs. Marlboro Lights SS smoke, (C) control vs. Advance Lights SS smoke, (D) control vs. Quest SS smoke. To determine if the treatments had a significant effect, means of the control and treatment group data collected at 80, 100, and 120 minutes were compared by a T-test. The p-values were all highly significant and are given on each graph.
attachment to Matrigel when compared to the untreated control (Fig. 5.3 A-D).
While control and treated colonies reached their maximum attachment by 2 hours,
the number of colonies that attached was significantly lower in all treatment groups
(20 to 60%) than in the control (70-90%). The percentages of inhibition for each
type of SS smoke are shown in Table 5.2. As was observed in our earlier work with
mESC (Linet al., 2009), the harm reduction brands were more inhibitory in this
assay than Marlboro Red, the conventional brand. Table 5.2 further shows that the
hESC were more sensitive to SS smoke exposure than the mESC.

<table>
<thead>
<tr>
<th></th>
<th>hESC</th>
<th>mESC a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marlboro Red (1.1mg/cig.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS (0.1PE)</td>
<td>39%</td>
<td>27%</td>
</tr>
<tr>
<td>Marlboro Lights (0.8mg/cig.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS (0.1PE)</td>
<td>60%</td>
<td>42%</td>
</tr>
<tr>
<td>Advance Lights (0.8mg/cig.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS (0.1PE)</td>
<td>76%</td>
<td>32%</td>
</tr>
<tr>
<td>Quest (No Nicotine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS (0.1PE)</td>
<td>69%</td>
<td>42%</td>
</tr>
</tbody>
</table>

a Data taken from Lin et al., 2009.

MS and SS smoke from conventional and harm reduction cigarettes inhibited
hESC colony growth at a non-cytotoxic dose (0.1PE)

To determine how conventional and harm reduction smoke solutions affect
proliferation of hESC, colonies were attached to Matrigel coated plates, allowed to
spread for 48 hours, then treated with control or smoke solution for an additional
48 hours during which time video data were collected on colonies in each group using the BioStation CT. All videos were first analyzed to determine if colonies grew, shrunk, or died during incubation (Fig. 5.4A). Although a few colonies died in most groups (Fig. 5.4 B, C), including the control, this percentage was very low in all groups, consistent with our prior data showing that 0.1PE is a non-cytotoxic dose. The percentage of colonies that underwent shrinkage during exposure was very low in the groups treated with MS smoke (Fig. 5.4B). However, the percentage of
shrinking colonies increased considerably in the SS treated groups (Fig. 5.4C), indicating that these colonies were able to survive at 0.1PE, but may have been losing some cells during the exposure interval. In the time lapse videos, some cells underwent apoptosis in colonies treated with SS smoke (Supplementary Figs 5.1-3), which may have contributed to shrinkage. Apoptosis was further confirmed in SS smoke treated colonies using the FLICA™ Poly Caspases Detection Kit (Fig.5.5).

Video data were next analyzed using a video bioinformatics method to determine how smoke treatment affected colony growth (Fig. 5.6). Kinetic analysis of colony growth using the video bioinformatics software showed that treatment of hESC colonies with 0.1PE of MS smoke did not alter the rate of growth when compared to the untreated control (Fig. 5.6A). Moreover, when analyzed by ANOVA, the mean percentage increase in colony size at the final frame was not significantly different among groups (p = 0.79) (Fig. 5.6B). In contrast, hESC colonies treated with SS smoke solutions had slower growth rates than the untreated control (Fig. 5.6C). Quest and Advance, two of the harm reduction brands, produced the greatest

![Image](image_url)
inhibition in rate of growth of the four brands tested. When the mean percentage increase in colony size was compared for the final frame at 48 hours, all four types of SS smoke solution significantly inhibited colony growth when compared to the untreated control (Fig. 5.6D). Again, both the Advance and Quest brands were the most inhibitory by this criterion. When growth characteristics were compared to our earlier data with mESC (Table 5.3), MS smoke was generally more inhibitory in
the mouse system than in the human. However, Marlboro Red, Advance Lights, and Quest SS smoke solutions were far more inhibitory in the hESC system than in the mouse (Table 5.3).

<table>
<thead>
<tr>
<th></th>
<th>hESC a</th>
<th>mESC b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Marlboro Red</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS (0.1PE)</td>
<td>-1%</td>
<td>13%</td>
</tr>
<tr>
<td>SS (0.1PE)</td>
<td>65%</td>
<td>39%</td>
</tr>
<tr>
<td><strong>Marlboro Lights</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS (0.1PE)</td>
<td>13%</td>
<td>17%</td>
</tr>
<tr>
<td>SS (0.1PE)</td>
<td>41%</td>
<td>84%</td>
</tr>
<tr>
<td><strong>Advance Lights</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS (0.1PE)</td>
<td>-17%</td>
<td>20%</td>
</tr>
<tr>
<td>SS (0.1PE)</td>
<td>85%</td>
<td>39%</td>
</tr>
<tr>
<td><strong>Quest (No Nicotine)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS (0.1PE)</td>
<td>-25%</td>
<td>32%</td>
</tr>
<tr>
<td>SS (0.1PE)</td>
<td>&gt;100%</td>
<td>76%</td>
</tr>
</tbody>
</table>

a Negative numbers indicate growth was not inhibited relative to the control.
b Data taken from Lin et al., 2009.
4. Discussion

Our study accomplished three goals: (1) to develop hESC-based toxicological assays, (2) to compare the toxicity of smoke from harm reduction and conventional cigarettes using hESC assays, and finally (3) to compare the response of human and mouse ESC to smoke from conventional and harm reduction cigarettes. Because embryos and fetuses are generally more sensitive to chemicals than adults, prenatal development is the best stage of the life cycle to use for estimating the risk which environmental chemicals present to human health (Grandjean, et al., 2007). While it is not possible to study human prenatal stages directly, embryonic stem cells, which are derived from the inner cell mass of blastocysts, can be used to model pre and post implantation embryos. Although assays with mESC have been developed and even validated (Genschow, et al., 2004, Spielmann, 2005), hESC have not been easy to adapt to toxicological testing. Therefore while attachment and proliferation endpoints worked well for evaluating the effects of cigarette smoke on mESC (Lin, Tran and Talbot, 2009), these assays were difficult to directly adapt to the hESC model. New approaches for using hESC in toxicological testing are being explored (Adler, et al., 2008), and as pointed out previously, quantitative analyses of microscopic video data would be a powerful technology for characterizing the effects of toxicants on dynamic cellular processes (Cervinka, et al., 2008). In this study, we introduced a method for evaluating chemical toxicity with hESC by collecting time-lapse video data using BioStation technology then mining
quantitative information from the videos either manually (attachment assay) or with video bioinformatics tools (colony growth). Combining video and quantitative analysis allows thorough investigation of the effects of chemicals on dynamic cellular processes through morphological, behavioral, and molecular endpoints and opens up the possibility of developing an unlimited number of powerful in vitro assays that can be used in the field of toxicology in the future. Furthermore, cellular assays, such as those introduced in this study, are relatively rapid and inexpensive, help to minimize animal usage, and provide data directly on a human model.

In developing the hESC model, we first distinguished cytotoxic and non-cytotoxic doses of smoke using morphological criteria and trypan blue staining. At 1 PE, MS and SS smoke solutions from all brands tested, except MS Quest, were cytotoxic, and SS smoke consistently produced stronger effects than MS smoke. In contrast, at 0.1PE, MS and SS smoke solutions were not cytotoxic for any brand tested.

To determine how PE doses are related to doses inhaled by smokers, the concentration of nicotine, a biomarker for smoke exposure, was compared in smoke solutions and human smokers. Nicotine levels in tissues, fluids, and matrices can be 2.9 (breast milk) to 87 (saliva) times higher than in plasma (Benowitz, et al., 2009, Dahlstrom, et al., 1990). In previous studies, nicotine concentrations in the plasma of active smokers were reported to range from 0.004 to 0.100ug/ml (Benowitz et al., 2009, Russel, et al., 1980). Based on a nicotine concentration at the approximate midpoint in this range (0.05 μg/ml), tissue nicotine concentrations in active
smokers can be estimated to be from 0.145 to 4.35 μg/ml. At 0.1PE, the nicotine concentration in Marlboro Red MS smoke was 0.3-0.6 μg/ml, which is well within the estimated tissue range of nicotine concentrations in active smokers.

Nicotine concentrations in the plasma of passive smokers vary depending on the amount of exposure per day and have been reported to range from 0.006 to 0.023 μg/ml (Dhar, 2004, Russell and Feyerabend, 1975). The estimated level of nicotine in tissues of passive smokers at the midpoint of the plasma range is 0.015 μg/ml. Based on the 2.9 to 87 fold increase of nicotine concentration in fluid and tissue vs plasma (Benowitz et al., 2009, Dahlstrom, Lundell et al., 1990), tissue nicotine concentrations in passive smokers can be estimated to be from 0.044-1.305 μg/ml. Nicotine concentration in SS smoke solutions made on our smoking machine was estimated from a previous study (Wong, et al., 2004) to be 0.2 μg/ml at doses of 0.1 PE, which is within the estimated tissue range of passive smokers. Since 0.1PE was non-cytotoxic and reasonably simulated doses to which both active and passive smokers are exposed, we used this concentration in our subsequent attachment and colony growth assays.

Attachment of cells to a substrate is very important in embryological development, and in the case of cultured hESC prevents the occurrence of apoptosis. Our results clearly showed that 0.1PE of SS smoke from all brands inhibited attachment of cells to Matrigel in the hESC system. Similar inhibition of hESC attachment occurred when 1.8 to 3.7 μM of nicotine was included in the culture medium, and this inhibition was reversible by the nicotine antagonist tubocurarine,
suggesting action was through a nicotine receptor (Zdravkovic, et al., 2008).

Nicotine caused similar inhibition of periodontal fibroblast attachment in culture (James, et al., 1999). However, factors in addition to nicotine must impair attachment since, in our study, Quest cigarette smoke solution did not contain nicotine, yet significantly inhibited attachment. Attachment of differentiated cultured cells is also affected by cigarette smoke. For example, attachment of bovine bronchial epithelial cells to fibronectin was inhibited by smoke condensate (Cantral, et al., 1995) and attachment of periodontal ligament fibroblasts to tooth roots was likewise significantly decreased when cells and roots were isolated from smokers vs. non-smokers (Gamal and Bayomy, 2002). Similar effects may occur in vivo during development as in utero exposure to smoke appears to increase the spacing between lung alveolar attachments as shown in infants that died from sudden infant death syndrome (Elliot, et al., 2003). Data in general support the conclusion that nicotine plus other chemicals in cigarette smoke inhibit attachment of embryonic and differentiated cells to substrates. Of particular concern in our study was the finding that SS smoke from harm reduction brands was a more potent inhibitor of attachment than SS smoke from a conventional brand.

Cell proliferation and growth are also essential and fundamental events during prenatal development. In our colony growth assay, non-cytotoxic doses (0.1PE) of MS smoke did not significantly affect growth over 48 hours. In contrast, 0.1 PE of SS smoke from all brands slowed colony growth rate and significantly inhibited the final size of colonies at 48 hours. The strongest effects in this assay
were produced by SS smoke from Advance and Quest cigarettes, two harm reduction brands. The reason that the harm reduction brands are more potent in this and the attachment assay is not known, but could be related to the source of the tobacco used in these products or methods used to cure the tobacco.

Other studies also support the conclusion that growth is negatively impacted by cigarette smoke. Condensate from Quest low-nicotine and nicotine-free cigarettes inhibited human bronchial epithelial cell growth and was more potent than condensate from a research cigarette (Chen, et al., 2008). The dose of the condensate used in the Quest study can not be directly compared to the doses of our smoke solution; however, because significant apoptosis was observed in the bronchial epithelium, doses of Quest smoke condensate may have been in the cytotoxic range. In the study by Chen et al, condensate from nicotine free-Quest inhibited proliferation more strongly than condensate from nicotine-containing Quest, an effect that was partially reversed by adding nicotine to condensate from nicotine-free Quest. In contrast to the Quest data (Chen et al., 2008), addition of nicotine alone to culture medium has been reported to increase the percentage of hESC undergoing apoptosis (Zdravkovic et al., 2008).

Relatively few studies have investigated harm reduction smoke, in particular SS smoke, in cellular based assays. One such study used explants of hamster oviducts to show that treatment with smoke from harm reduction cigarettes (Advance Lights and Omni Lights) significantly inhibited oocyte pick-up rate and smooth muscle contraction rate with SS smoke generally being more potent than MS
smoke (Riveles, et al., 2007). Mouse lungs exposed to light cigarette smoke exhibited a decrease in tissue inhibitor of metalloprotease-2 and an increase in matrix metalloprotease-2 expression, suggesting an imbalance in extracellular matrix formation. As a consequence of light cigarette smoke exposure, these mice developed emphysema and increased levels of NFκB which increased inflammatory response in the lungs (Valenca et al., 2006). When taken together, the limited work done with cellular and animal models supports the idea that smoke from harm reduction cigarettes can impair a spectrum of biological processes. Moreover, SS smoke from harm reduction brands is usually more toxic than MS smoke from the same cigarette or than SS smoke from conventional cigarettes. This information should be made available to potential users of harm reduction cigarettes and should be taken into account when establishing policies regarding their sale and use.

Because animal models may not accurately predict toxicity for humans, we compared the effects of cigarette smoke on mESC from our previous study (Lin et al., 2009) to the results of this study, and found that, in general, cells from both species responded similarly to cigarette smoke. However, the sensitivity of the two species was distinctly different. Although SS smoke from both harm reduction (0.1PE) and the conventional brand inhibited cell attachment, the negative impact of SS smoke on hESC (60-76%) was greater than on mESC (30-42%). In the proliferation assay, mESC were more sensitive than hESC colonies to MS smoke. However, SS smoke had a greater negative impact on colony growth for hESC (41-100%) than for mESC (39-84%). These data demonstrated that while the effects of smoke are similar
between species, the sensitivity in specific assays varied between species and that it
would therefore be preferable to use hESC when assessing the potency of smoke or
other environmental toxicants.

In conclusion, this study introduces a rapid, high-content, *in vitro*
toxicological assay based on hESC as a novel model for early embryos. This assay
provides quantitative data on dynamic cellular processes during exposure to
potential toxicants in incubation conditions that support optimal cell growth and
survival. The assay which combines time-lapse video data with video bioinformatics
tools could easily be expanded in the future to provide more endpoints and to
evaluate other chemicals. Data obtained with this assay demonstrated that at a non-
cytotoxic dose of SS smoke, cell attachment to Matrigel and growth of hESC colonies
are inhibited. Not only was SS smoke consistently more potent than MS smoke but
also SS smoke from the harm reduction brands was generally more potent than SS
smoke from the conventional brand. These data demonstrate a need to monitor all
components produced by harm reduction products and not assume that because
carcinogens are reduced in MS smoke the overall product is necessarily safer than a
conventional counterpart. Moreover, comparison to similar data obtained with
mESC revealed that both the human and mouse ESC detected the toxicity of SS
smoke from harm reduction products; however, the human model was more
sensitive to SS smoke from two harm reduction brands than the mouse model. Again
this reinforces the desirability of using hESC for toxicological studies to obtain a
better estimate of the risk posed directly to humans.
5. References


CONCLUSIONS

Cigarette smoke, a major environmental toxicant, is a complex mixture of over 4,000 chemicals, and tobacco-related disease is the leading cause of death in the United States. In addition to death, smoke has been linked to a number of reproductive problems. Although epidemiological, in vivo (animal models), and in vitro assays (cell models) have consistently shown that smoking adversely affects reproduction, little is known about the effects of cigarette smoke on prenatal development, in particular the pre-implantation stages. Heightened awareness to the dangers of cigarette smoke has prompted interest in harm reduction tobacco products that could lessen the level of disease caused by tobacco use (Hatsukami, et al., 2004). In general, harm reduction tobacco products have lower amounts of tar, nicotine, and/or carcinogens, and are therefore often presumed to be safer than conventional cigarettes (Warner, 2005). However, this idea has been questioned largely due to an increasing number of scientific reports, most dealing with MS smoke, that show detrimental effects of harm reduction tobacco products on health and development (Valenca et al., 2004, Chen et al., 2006). Therefore, in this study, we were interested in comparing the effects of MS and SS smoke from conventional (Marlboro Red) and harm reduction (Marlboro Lights, Advance Lights, and Quest) cigarettes on ESC.

Since ESC are derived from the inner cell mass of blastocysts, they are an excellent model for studying human embryotoxicity. In this study, several toxicity assays have been developed to evaluate cell attachment, survival, proliferation,
growth, and apoptosis of mESC and hESC. The goals of this study were: (1) to use ESC as a model for pre-implantation embryos in toxicity assays, (2) to investigate the effects of MS and SS smoke on dynamic processes in ESC, (3) to compare the effects of harm reduction and conventional cigarettes on ESC, and (4) to compare the responses of the mouse and human ESC models to cigarette smoke.

In the mouse study, the effects of conventional and harm reduction cigarette smoke on mESC attachment and proliferation were evaluated. mESC were incubated in smoke solutions and counted at 6 hours (attachment assay) and 24 hours (proliferation assay). All experiments were done using D3 mouse ESC. mESC responded negatively to both MS and SS smoke solutions which inhibited cell attachment and proliferation dose dependently. For all brands, SS smoke was more inhibitory than MS smoke, and interestingly, both MS and SS smoke from harm reduction cigarettes were consistently more potent than smoke from the conventional brand. Formation of embryoid bodies (EB) was also inhibited at concentrations of 1PE (1 Puff Equivalent = the smoke from 1 puff that dissolves in 1 ml of medium), and the rate of EB attachment and spreading was also retarded. Gene expression analysis revealed that smoke treated EB exhibited altered expression patterns. The pluripotency marker Rex-1 was down-regulated by MS smoke at 0.1PE, and both MS and SS smoke up-regulated endodermal and mesodermal markers when compared to the control. To confirm the validity of mESC as a model for studying embryotoxicity in pre-implantation embryos, morula stage embryos were collected and treated with MS or SS smoke. Both MS and SS
smoke treatments induced apoptosis in blastomeres with SS smoke being more potent than MS smoke. This study showed that cells derived from the inner cell mass were inhibited from attaching and growing by both MS and SS smoke solutions, that SS smoke was more potent than MS smoke, and that harm reduction brands were more potent than a conventional brand in most assays.

Although fast and efficient toxicological tests for attachment and proliferation were established for the mouse model, the hESC system was not as easy to adapt to similar assays due to differences in the two culture systems. To overcome these challenges, we introduced a new assay that uses video bioinformatics tools to mine data from time-lapse videos. Video bioinformatics is the automated processing, analysis, understanding, and data mining of biological spatio-temporal data extracted from microscopic videos. This method enabled us to use dynamic cellular processes as endpoints in the toxicological assays. Time-lapse videos of colonies were collected using the BioStation technology over 48 hours, and hESC attachment and colony growth were evaluated by hand (attachment) or with the CL-Quant software (colony growth). For the latter, custom-developed algorithms (recipes) were created in the software with three subset routines: (1) segmentation, (2) enhancement, and (3) measurement recipes, and measurements of colony size were plotted over time. Truthfulness of the CL-Quant recipes were validated using the Adobe Photoshop software by comparing colony area measured with both software packages. The use of video bioinformatics tools greatly enhances analysis
and makes data mining faster and more reproducible than when done manually by a human.

With the new BioStation technology and video bioinformatics tools, we then effectively evaluated the effects of harm reduction and conventional cigarette smoke on hESC. We established that both MS and SS smoke were cytotoxic at 1PE but not at 0.1PE, using morphological and trypan blue assays. At 1PE, treated colonies often exhibited granular morphology and detached cells from the colonies. In addition, dead cells were verified with trypan blue staining, and caspase detection kits, which showed that 1PE of both MS and SSsmokesolutions induced apoptosis in hESC. The effect of MS or SS smoke on hESC attachment and growth were then evaluated using the non-cytotoxic dose (0.1PE), and colonies were followed in the BioStation IM (attachment) or the BioStation CT (colony growth). In the attachment assay, 0.1PE SS smoke inhibited colony attachment significantly when compared to the control, and harm reduction SS smoke (60-79%) was more inhibitory than the conventional brand (39%). In the colony growth assay, time-lapse videos of control and treated colonies were evaluated using CL-Quant software, and the results showed that all four brands of SS smoke inhibited colony growth significantly. Similar to the mESC data, harm reduction cigarette SS smoke (Advance Lights, 85%, and Quest ≥100%) was more potent than conventional brand (65%). In contrast, MS smoke from all four brands of cigarettes was not inhibitory to hESC colony growth at 0.1PE. When data from mESC and hESC systems were compared, hESC were far more sensitive to SS smoke treatment than mESC.
In conclusion, this study successfully established predictive embryotoxicity assays that thoroughly investigated the toxicological effects of conventional and harm reduction MS and SS smoke on early development by using ESC as a novel model for pre-implantation embryos. Here, a number of dynamic cellular processes (i.e., cell attachment, survival, proliferation, and apoptosis) were examined and quantified during exposure to environmental toxicants in optimal cell growth and survival conditions. These assays revealed that both MS and SS cigarette smoke were toxic to mESC and hESC, but generally, SS smoke was more potent than MS smoke. In addition, more importantly, MS and SS smoke from harm reduction cigarettes were generally more inhibitory than smoke from the conventional brand. Inhibitory effects of cigarette smoke on mESC and hESC attachment, survival, and growth could potentially explain the observations made in epidemiological studies where infants suffer from a number of health issues due to smoke exposure. Data obtained in this study revealed the importance of monitoring all chemical components of MS and SS smoke from harm reduction cigarettes before making the assumption that these cigarettes are safer because they have lower concentrations of toxins and carcinogens. Furthermore, although data from the mESC and the hESC studies showed that both model systems detected toxicity of cigarette smoke, hESC were more sensitive to smoke solutions than mESC. These findings demonstrate that it is important to evaluate chemicals using human models to prevent species-specific responses to stimuli.
References


APPENDIX
LITERARY REVIEW

CIGARETTE SMOKE'S EFFECT ON FERTILIZATION AND PRE-IMPLANTATION DEVELOPMENT: ASSESSMENT USING ANIMAL MODELS, CLINICAL DATA, AND STEM CELLS
Abstract

Numerous studies have repeatedly shown that women who smoke experience problems establishing and maintaining pregnancies, and recent work has further demonstrated that the in utero effects of smoke may not be manifested until months or even years after birth. The purpose of this review is to examine the recent literature dealing with the effects of cigarette smoke on the earliest stages of human prenatal development. Studies in this area have included the use of animal models, patients undergoing in vitro fertilization, and embryonic stem cell models. Events leading to fertilization such as cumulus expansion, hyperactivation of sperm motility, and oocyte pick-up by the oviduct have all been shown to be impaired by smoke exposure in animal models. Steps crucial to fertilization such as the sperm’s acrosome reaction and sperm binding to the zona pellucida are likewise inhibited by cigarette smoke. Preimplantation embryos and stem cells that model embryos show a number of adverse responses to smoke exposure including poor adhesion to extracellular matrices, diminished survival and proliferation, and increased apoptosis. The currently existing literature demonstrates that the earliest stages of human development are sensitive to smoke exposure and indicates that further work is needed to address the details of these effects.
1. Introduction

Two general classes of cigarette smoke exist, mainstream smoke (MS) is the puff of smoke inhaled by active smokers, while sidestream smoke (SS) burns off the end of a cigarette and is inhaled by passive smokers. SS smoke, which is the major component of environmental tobacco smoke, is also referred to as secondhand smoke (EPA, 2005; USDHHS, 2006). Cigarette smoke is a complex colloidal mixture containing well over 4,000 chemicals (EPA, 1992). Nicotine is probably the most thoroughly studied bioreactive chemical in cigarette smoke (Benowitz, 2008), but other chemicals that are known toxicants and carcinogens have likewise been studied (Hoffmann, Djordjevic & Hoffmann, 1997; Jenkins, Guerin & Tomkins, 2000). These include, for example, tar, heavy metals such as cadmium and lead, polycyclic hydrocarbons (PAHs), phenol, benzene, carbon monoxide, nitrosamines, and hydrogen cyanide. In addition, other chemicals that have not traditionally been recognized as toxic, such as pyridines, pyrazines, and phenols, have recently been shown in various biological assays to produce adverse effects at relatively low doses (Ji et al., 2002; Melkonian et al., 2003; Talbot & Riveles, 2005). Given the complexity of the mixture of chemicals in burning tobacco, it is not surprising that most chemicals in smoke have not been studied thoroughly and the interaction of these chemicals is not well understood.

A number of epidemiological studies have shown that smoke exposure affects reproductive processes and that inhalation of either MS or SS smoke during pregnancy can cause adverse outcomes (Castles et al., 1999; DiFranza, Aligne &
Weitzman, 2004; Hegaard et al., 2006; Kharrazi et al., 2004; Rogers, 2008; Stillman, Rosenberg & Sachs, 1986). For example, the birth weight of fetuses born to mothers who smoke during pregnancy has repeatedly been shown to be lower than that of fetuses from non-smoking mothers (Rogers, 2008; Windham et al., 2000). Moreover, this relationship holds for in utero exposure to SS smoke, although the decrease in weight is not as large as for MS exposure (Goel et al., 2004; Windham et al., 2000). Other disturbing consequences of smoke exposure during pregnancy include increased incidences of ectopic implantation, stillbirth, structural malformations, and placental malfunction (Rogers, 2008; Saraiya et al., 1998; Shiverick & Salafia, 1999). Additionally, normal appearing offspring exposed to smoke in utero may be victims after birth of sudden infant death syndrome, obesity, and decreased cognitive and respiratory development (Dwyer, Broide & Leslie, 2008; Maritz, 2008; Rogers, 2008).

In spite of numerous epidemiological and animal studies done previously on smoking during pregnancy, we know relatively little about how smoke affects the earliest stages of prenatal development. The purpose of this review is to consolidate recent information addressing the effects of cigarette smoke exposure on events beginning with ovulation of the oocyte and extending through blastocyst formation. We will include recent data obtained using embryonic stem cells as models for the pre-implantation stage of development.
2. Ovulation Through Fertilization

Background

Ovulation and fertilization are complex processes requiring multiple steps to achieve evacuation of the oocyte cumulus complex from the mature follicle and subsequent fusion of a sperm and oocyte (Richards, 2005; Talbot & Riveles, 2005; Talbot, Shur & Myles, 2003; Yanagimachi, 1984), and therefore present a variety of potential targets for cigarette smoke. These targets include expansion of the oocyte cumulus complex, formation of a rupture site, escape of the oocyte from the follicle, transport of the gametes to the ampulla of the oviduct where fertilization occurs, sperm penetration through the cumulus, sperm binding to the zona pellucida and induction of the acrosome reaction, sperm penetration of the zona pellucida, and gamete membrane fusion (Figure 1). The effects cigarette smoke on some of these processes and the cells associated with them have been examined experimentally in animal systems, and additional insight has been gained through studies done at in vitro fertilization (IVF) clinics working with human gametes. Cumulus expansion and ovulation: Following the LH surge, the cumulus cells surrounding the maturing oocyte secrete a massive extracellular matrix that is rich in hyaluronic acid stabilized by cross-linked molecules of inter-α-trypsin inhibitor (Chen et al., 1996). As the matrix hydrates, it causes the cumulus cells to move apart from each other creating a large oocyte cumulus complex (OCC) that is ultimately ovulated from the mature follicle. This process, called cumulus expansion, is important as it facilitates the escape of the oocyte from the antrum of the follicle during ovulation (Talbot,
1983), and the secreted matrix enables the OCC to stick to the surface of the infundibulum of the oviduct from which it migrates into the ampulla of the oviduct where fertilization occurs (Lam et al., 2000; Talbot, Gieske & Knoll, 1999). Expansion also separates cumulus cells from each other clearing a pathway of low resistance through which sperm can eventually penetrate and reach the zona pellucida (Talbot et al., 1985). The importance of the cumulus matrix can not be overstated. Mice that are deficient in the matrix stabilizing proteins, inter-α-trypsin inhibitor and tumor necrosis factor-induced protein-6, do not assemble functional matrices and are infertile (Fulop et al., 2003; Zhuo et al., 2001). Various studies have shown that the cumulus matrix is sensitive to the chemicals in cigarette smoke. Porcine oocyte cumulus complexes failed to expand properly when exposed in vitro to cadmium, anabasine, and nicotine (chemicals found in cigarette smoke), and additionally, nicotine and cadmium inhibited synthesis and assembly of hyaluronic acid, the major component of the cumulus matrix (Mlynarcikova et al., 2004; Vrsanska et al., 2003). In smokers, diminished functionality of the cumulus cells could be due to DNA damage which increases significantly in human cumulus cells from smokers vs. non-smoking control patients undergoing IVF (Sinko et al., 2005).

A defect in cumulus expansion could make evacuation of the antrum difficult and may prevent some oocytes from escaping the follicle during ovulation (Talbot, 1983). Indeed, evidence from animal experiments indicates that the number of ovulating follicles decreases when exposed to smoke or smoke constituents. For example, hamsters that inhaled MS smoke for 30 days prior to and for the first 7
days during pregnancy showed a dose dependent decrease in the number of corpora lutea (which would be indicative that ovulation was inhibited) (Magers et al., 1995). Moreover, benzo(a)pyrene, a chemical in cigarette smoke, decreased the number of corpora lutea in pregnant mice (Swartz & Mattison, 1985). In human IVF clinics, oocytes are normally aspirated, not ovulated from follicles. Nevertheless, in one study involving IVF patients, various parameters associated with ovulation were found to be adversely affected in smokers when compared to non-smoking controls (Van Voorhis et al., 1992). Defects in cumulus expansion coupled with difficulty in achieving ovulation could be factors in the reported decrease in the fertility of smokers (USDHHS, 2001).

**OCC Transport into the Oviduct**

OCC complexes, once released from the tertiary follicle, are picked up by the oviduct and rapidly transported into the ampulla by beating cilia on the outer surface of the infundibulum of the oviduct (Talbot & Riveles, 2005). OCC pick-up requires adhesion between the tips of the cilia and the cumulus matrix, and factors that increase or decrease adhesion inhibit the rate of pick-up (Lam et al., 2000; Mahi-Brown & Yanagimachi, 1983; Norwood & Anderson, 1980). Inhalation of either MS or SS smoke prior to and during pregnancy increased the number of blebs on the oviductal epithelium of hamsters indicating smoke targets these cells (Magers et al., 1995). Both MS and SS smoke solutions inhibited ciliary beat frequency and oocyte pick-up rate in experiments using hamsters oviductal explants (Knoll & Talbot, 1998). Fractionation and screening of smoke solutions using solid
phase cartridges lead to the identification of about 30-40 chemicals that were inhibitory in the beat frequency and oocyte pick-up assays (Riveles et al., 2003; Riveles et al., 2004; Riveles, Roza & Talbot, 2005). These chemicals were mainly pyridines, pyrazines, and phenols, and some were inhibitory at very low doses. Failure of the OCC to be picked up at the normal rate could lead to ectopic implantation, which increases significantly in smokers, or failure of fertilization. Pick-up rate was subsequently shown to be slowed in smoke treated groups because the components in smoke increased the adhesion between the cilia on the oviduct and the matrix between cumulus cells (Gieseke & Talbot, 2005). The oviduct was further shown to be more seriously affected by smoke treatment than the OCC.

**Fertilization**

With the widespread use of *in vitro* fertilization (IVF) to treat infertility, the opportunities to collect data on reproductive processes surrounding fertilization are vast. Not surprisingly, numerous studies have been done in which various reproductive parameters including fertilization rate have been compared in smokers and non-smokers undergoing fertility treatment. Many of these studies have been recently examined in a meta-analysis (Waylen et al., 2009) and in several reviews (Cooper & Moley, 2008; Soares & Melo, 2008). While there is not a consensus among these studies, some interesting trends are emerging. Soares and Melo (2008) concluded in their review that fertilization rate decreased in smokers, while Waylen et al (2009) concluded that fertilization rate was not different in most studies included in their meta-analysis. Since patients in IVF clinics are undergoing
treatment for infertility, the non-smoking controls in these studies may not be robust enough to detect differences that would exist in the population at large or in a fertile population. In any case, very few IVF studies directly access the detailed events of fertilization, such as sperm penetration of the cumulus matrix, sperm binding to the zona pellucida, induction of the acrosome reaction, penetration of the zona, gamete membrane fusion, and establishment of blocks to polyspermy in the context of smoke exposure, and such studies would be difficult to perform in humans in vitro or in vivo.

Animal studies, which use controlled experimental conditions and eliminate confounding variables present in IVF work, have been done on some aspects of fertilization and provide insight into smoke’s effect on events leading to and including fertilization. Inhalation of cigarette smoke by rats for 10 weeks prior to performing in vitro fertilization decreased fertilization rates (Yamamoto et al., 1998). Although the specific cause of the problem was not determined in this study, fertilization was done in vitro suggesting that smoke exposure interfered with processes involved in fertilization and not factors involving the female reproductive tract (Yamamoto et al., 1998). Since nicotine and smoke exposure alter the properties of the cumulus matrix (Gieseke & Talbot, 2005; Vrsanska et al., 2003), it is certainly possible that sperm penetration though the cumulus is affected by smoking. Exposure of Rhesus monkey sperm to environmental tobacco smoke decreased their motility and ability to undergo hyperactivation when exposed to dbcAMP or caffeine (Hung et al., 2007). This could be due to damaged mitochondria
in sperm treated with environmental tobacco smoke (Hung et al., 2007). In an interesting trial using patients undergoing in vitro fertilization, sperm from heavy smokers produced more female than male embryos, and this correlated with better performance of X bearing sperm in the swim up test, again suggesting that smoking affects sperm motility, which in turn affects sex ratio (Viloria et al., 2005). Although acrosin levels in sperm from smokers and controls are not different, the inducibility of the human sperm acrosome reaction by ionophore A23187 is significantly lower in sperm from smokers than from fertile controls (El Mulla et al., 1995), which could impair sperm penetration through the zona and subsequent gamete membrane fusion. The difficulty in inducing acrosome reactions in smoker’s sperm could be related to the level of calcium inside sperm. Calcium, which is required for induction of the acrosome reaction (Talbot et al., 2003), is reduced in boar sperm treated with the extractable-respirable particulates of environmental tobacco smoke (Zhou et al., 2000). Primate sperm treated with environmental tobacco smoke did not bind as well as untreated sperm to the zona pellucida (Hung et al., 2007). At the higher dose tested, a higher percentage of bound sperm were acrosome reacted, which could be due to induction of “false” acrosome reactions by toxic levels of smoke treated medium. The zona pellucida in both active and passive human smokers is significantly thicker than in non-smokers (Shiloh et al., 2004). Although not yet directly investigated, this increase in zona thickness could retard sperm penetration and decrease fertilization rate.
3. Preimplantation Development

**Animal studies on embryo movement though the oviduct**

Movement of preimplantation embryos through the oviduct occurs in a precisely timed manner. Anything that alters the rate of transport can present complications to the establishment of a normal pregnancy. Moving too quickly could result in expulsion of the embryo from the female tract prior to implantation, while retarded movement may result in implantation in an ectopic site. Animal studies have shown that inhalation of either MS or SS smoke retards movement of hamster preimplantation embryos through the oviduct (DiCarlantonio & Talbot, 1999). The doses that were effective in this study are similar to those found in human smokers based on measured values of cotinine in the serum of exposed hamsters. Moreover, the reduction in transport rate of the preimplantation embryos was correlated with decreased rates of contraction of the oviduct smooth muscle, suggesting that smoke exposure reduces muscle contractions thereby slowing transport rate. Nicotine alters the contractility of primate oviducts and may be one of the active factors in smoke that affects oviductal contractility (Neri & Marcus, 1972). The well documented increase in ectopic pregnancy occurring in women who smoke during pregnancy could be related to a decrease in embryo transport rate through the oviduct (Handler et al., 1989; Saraiya et al., 1998).

**Studies Using Embryonic Stem Cells (ESC)**

Embryos and fetuses are generally more sensitive to environmental toxicants than adults. In 2007, toxicologists released the Faroes Statement emphasizing the
importance of studying prenatal development when evaluating environmental toxicants and drugs due to the likelihood that these stages will be the most sensitive of the entire life cycle (Grandjean et al., 2007). However, relatively little is known about the direct effects of environmental toxicants on early human pre-implantation embryos due to the ethical limitations of using human embryos for testing. Most prenatal toxicological studies have been done with animal models such as mice and rats, which have served us well, but which may not respond similarly to humans. Recently, toxicologists have turned to embryonic stem cells (ESC) as models for prenatal development. Pluripotent ESC, which are derived from the inner cell mass of blastocysts (Martin, 1981; Thomson et al., 1998), self renew indefinitely, and have the ability to produce daughter cells that can differentiate into all the cells of an embryo. Thus embryonic stem cells themselves represent a model of the preimplantation embryo, and embryonic stem cells which form embryoid bodies and differentiate model post implantation development. The most thoroughly developed model based embryonic stem cells and the only one that has been thoroughly validated is the “embryonic stem cell test”developed in Europe (Chapin et al., 2008; Croxatto & Villalon, 1994; Davila et al., 2004). While originally based on differentiation of contracting cardiomyocytes, this assay has undergone continual improvements to include molecular endpoints (Seiler et al., 2004; zur Nieden, Kempka & Ahr, 2004) and also to reduce the time required to perform the assay (Buesen et al., 2009). However, the “embryonic stem cell test”has not yet been adapted to hESC, and species variations in response to toxicants and the accuracy of
its predictivity are of concern and can result in incorrect classification of substances.

Undifferentiated embryonic stem cells *per se* have recently been used by several labs to model pre-implantation development in studies involving cigarette smoke or nicotine. In a study using mESC, nicotine doses that bracketed those found in human smokers were found using quantitative RT-PCR to increase expression of Oct-4 and Rex-1, two genes associated with pluripotency (Zhang et al., 2005). This effect could be prevented by tubocurarine, a nicotinic acetylcholine receptor antagonist. In a more recent study involving hESC, nicotine, again at doses that would be found in the reproductive tract of women who smoke during pregnancy, affected various endpoints (Zdravkovic et al., 2008). Nicotine inhibited adhesion of human ESC to extracellular matrix (Matrigel), an effect that was also blocked by tubocurarine. Moreover, nicotine treatment may have blocked attachment of suspended cells by interfering with integrin α5/β1, which was reduced in suspended cells treated with nicotine. Treated cells also had numerous small cytoplasmic vacuoles which disappeared when nicotine was washed out. A higher percentage of both attached and unattached hESC underwent apoptosis when treated with nicotine than when incubated in control medium, and this effect was reversed by tubocurarine in the unattached population. Induction of apoptosis by nicotine had been observed previously in post-implantation mouse embryos that were cultured and exposed to nicotine *in vitro* (Zhao & Reece, 2005). In addition to nicotine, a PAH in cigarette smoke (7,12-dimethylbenanthracene or DMBA) has also been shown to produce apoptosis in pre-implantation mouse embryos (Detmar
et al., 2006), indicating that multiple chemicals in the complex milieu of cigarette smoke may be inducers of apoptosis in the early phase of development. The effect of nicotine on expression of pluripotency markers is not yet clear. In contrast to the study using mESC (Zhang et al., 2005), hESC pluripotency markers appeared to decrease when cells were exposed to nicotine (Zdravkovic et al., 2008). The reason for this discrepancy is not known, but could be due to species differences (mESC vs. hESC) or culture conditions (mESC experiments were done on feeder layers while those with hESC were done directly on Matrigel). Overall, nicotine has been shown to affect a number of endpoints for hESC, and the reversibility of some of these effects by tubocurarine suggests that hESC have a receptor for nicotine.

In addition to nicotine, smoke solutions from various commercial cigarettes have also been tested for their effects on mESC attachment to gelatin, proliferation, survival, and apoptosis (Lin, Tran & Talbot, 2009). In this study, the toxicity of MS and SS smoke from traditional and harm reduction cigarettes was compared. Attachment and proliferation of ESC were inhibited dose dependently by treatment with MS or SS smoke solutions. On a puff for puff basis, SS smoke was more inhibitory in these assays than MS smoke. However, removing the filter from MS smoke increased its inhibitory effects significantly. Unexpectedly, in most assays, SS smoke from harm reduction brands (Marlboro Lights, Advanced Lights, and Quest) was more potent than SS smoke from the traditional brand (Marlboro Red), indicating the need for further evaluation of smoke from harm reduction products. Detection of activated caspases confirmed that smoke from all cigarette brands
induced apoptosis in ESC populations. The results of the ESC assays were verified with actual mouse pre-implantation embryos. Activated caspase 3 and 7 were detected in blastomeres of young mouse embryos after smoke treatment for 1 hour, demonstrating apoptotic activity within the inner cell mass.

Survival, attachment, proliferation, and apoptosis are important processes necessary for normal prenatal development. In the above studies with stem cells, attachment to a protein substrate (gelatin or Matrigel) was inhibited by whole smoke solutions (mESC) or by nicotine (hESC). Failure of cells to attach to extracellular matrices during early embryonic development could compromise subsequent steps in development, such as germ layer formation. Survival and proliferation of inner cell mass cells are likewise critical for proper embryo and fetal growth. In the mouse and human ESC models, treatment with nicotine (human) or smoke solution (mouse) increased the number of apoptotic cells in culture. While apoptosis occurs normally during prenatal development, too much apoptosis can impair development, leading to congenital defects (Zhao & Reece, 2005) or embryo death (Detmar et al., 2006). Having a lower number of viable cells in the inner cell mass could also ultimately lead to lower fetal birth weights, as are seen in pregnant women who smoke (Hegaard et al., 2006; Windham et al., 2000). The idea that smoking impairs fetal growth by acting at the preimplantation stage of development is further supported by an in vivo study in which inhalation of mainstream and sidestream smoke by mice, at levels simulating human exposure, during days 1-5 of pregnancy, was sufficient to significantly decrease birth weight (Esposito et al.,
2008). Interestingly, smoke exposure during days 6-18 of pregnancy did not affect birth weight, further emphasizing the sensitivity of the preimplantation embryo to smoke. Taken together, current data support the idea that the preimplantation embryo is adversely affected by cigarette smoke exposure and that women contemplating pregnancy should avoid exposure to smoke.
4. Conclusions

Although relatively few studies have addressed the specific effects of smoke on the earliest stages of human reproduction, there is sufficient information in the literature to conclude that smoking adversely cumulus expansion, gamete transport, fertilization, and in pre-implantation development (Fig. 1). These effects could hinder the establishment of pregnancies, e.g. due to decreasing fertilization rates and could also impair growth and development of embryos during and after implantation, e.g. by diminishing the number of cells contributing to the embryo.

The use of hESC as models of pre- and post implantation development should help further clarify the precise effects of smoke exposure on prenatal development in humans and have the further bonus of minimizing animal usage and eliminating concern about inter-species differences.

Fig. 1: Schematic diagram showing the early developmental events that are affected by cigarette smoke.
5. References


contraction in oviducts of hamsters (*Mesocricetus auratus*). *Biol Reprod* 61, 651-6.


